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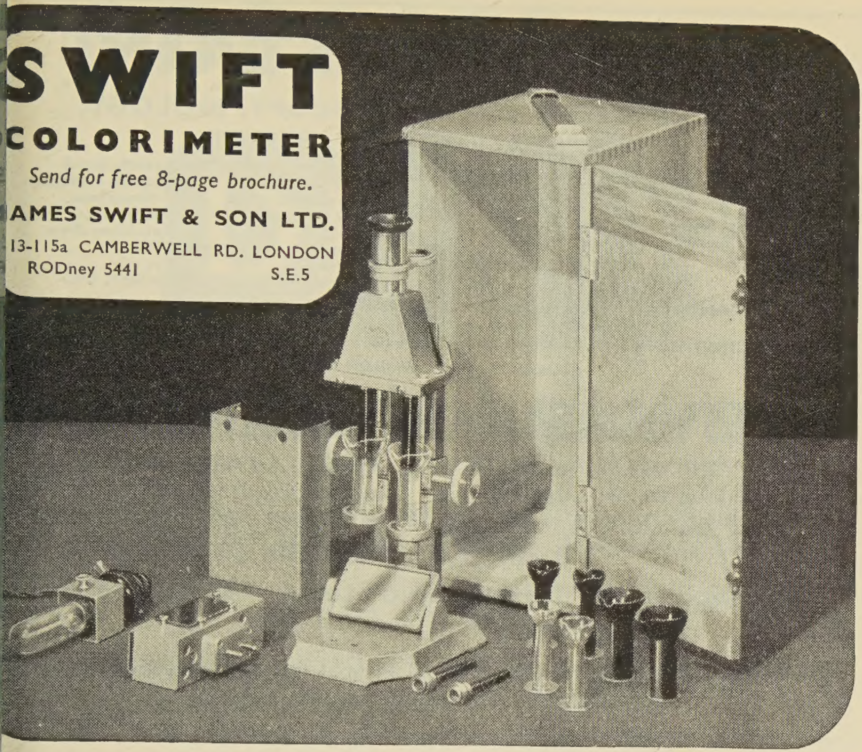

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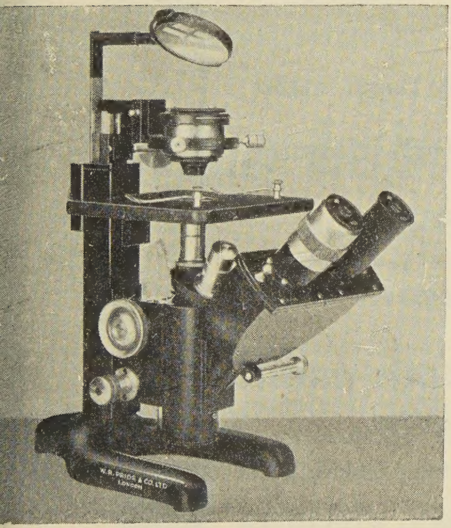

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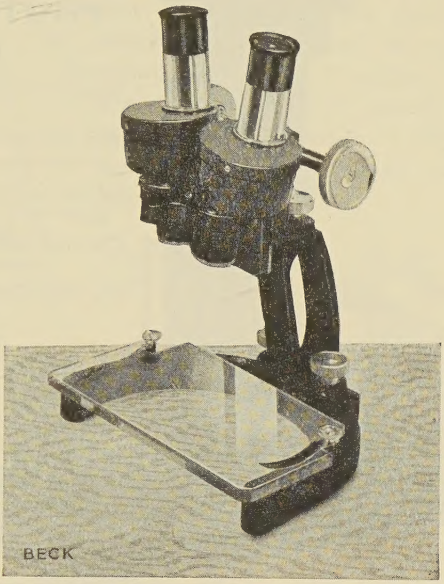

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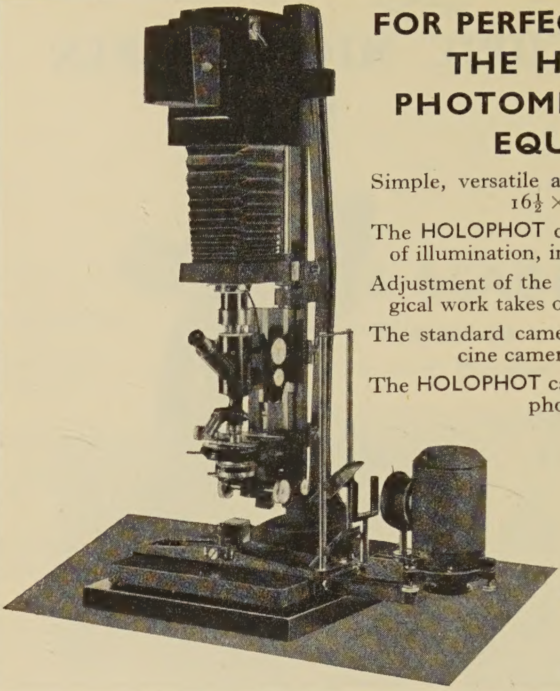

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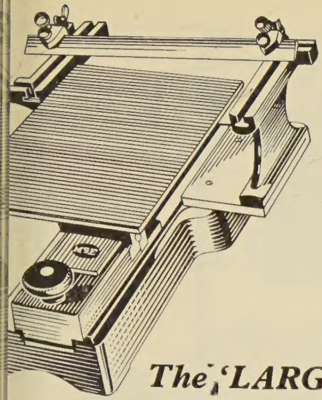

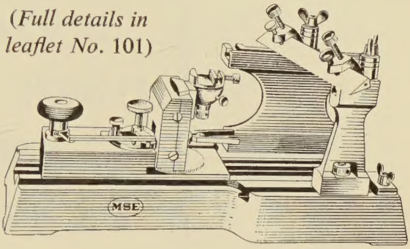

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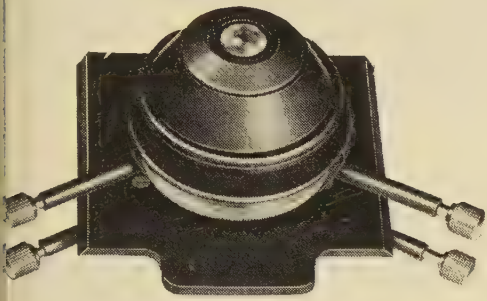

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The Botryoidal and Vaso-fibrous Tissue of the Leech *Hirudo medicinalis*

By S. BRADBURY

n *the Cytological Laboratory, Department of Zoology, University Museum, Oxford; present address, Department of Human Anatomy, South Parks Road, Oxford*)

With one plate (fig. 3)

SUMMARY

he botryoidal and vaso-fibrous tissues of the leech *Hirudo medicinalis* have been ied morphologically and histochemically. It is concluded that the cells forming e tissues represent cells of the vessel and sinus walls which have swollen as a conence of the accumulation of pigment granules in their cytoplasm. This swelling is h more pronounced in the cells of the botryoidal tissue. These tissue systems are in munication with each other and with the contractile and non-contractile haemoh vessels.

part from the pigment granules, few cytoplasmic inclusions were seen in the cells e botryoidal tissue, but numerous granules are present in the vaso-fibrous cells: e granules are about 1 μ in diameter and contain phospholipid. The brown pigt of the botryoidal tissue seems to consist of a masked iron compound, which ably occurs in combination with the protein which forms the framework of the ent granule; the pigment of the vaso-fibrous tissue is green, and contains no but reacts to the tests for bile pigments.

hese tissues are considered to represent 'kidneys of accumulation' for the iron and ile pigment which probably represent the by-products of a breakdown of the d used as food. The amount of botryoidal tissue is significantly greater in old than ung leeches.

INTRODUCTION

THE existence of two main types of pigmented tissue in *Hirudo medicinalis* and the other gnathobdellid leeches has been known for well over ntury. There has, however, been little agreement on either the nature of e tissues or their function; many divergent opinions are to be found in the ature and the most important of these are considered in chronological r in the following historical survey. Although many workers have studied e pigmented tissues by the standard methods of histology, it would seem nothing has been published on the chemical composition of the pigment. the principal purpose of this paper to fill this gap in knowledge.

HISTORICAL SURVEY

randt and Ratzeburg (1829) were among the first to mention the type of ented tissue which is now known as botryoidal tissue. These authors

rterly Journal of Microscopical Science, Vol. 100, part 4, pp. 483–498, Dec. 1959.]

were of the opinion that it was hepatic in character, a view which has not be supported by any subsequent studies. Gratiolet (1862) called the same tiss *réseaux variqueux* and thought it to be part of the vascular system. In t following year, Leuckart (1863), studying the anatomy of the leech by mea of transverse sections, thought that it represented a mass of deeply lyi epidermal glands.

Leydig (1857) had already expressed his opinion that the pigmented tiss was a form of connective tissue, most directly comparable to the fat-body arthropods. It was not until the studies of Ray Lankester (1880) that t tissue was investigated systematically. He named it 'botryoidal tissue' becau of its resemblance to a bunch of grapes (from the Greek βότρυς, a bunch grapes), and considered that it consisted of a plexus of 'haematophoro vessels', some of which formed blind diverticula, with walls swollen by t enlargement of the individual cells which constituted them. It was these ce which accumulated the brown pigment granules characteristic of the tiss Lankester states that the system is in direct communication with the cap laries of the blood-vessels and with the other type of pigmented tissue connective-tissue origin, which he termed 'vaso-fibrous', presumably becau of the similarity in form between it and the connective-tissue fibres. Four yea later Bourne (1884), in his monograph on the anatomy of the Hirudinea, ca to a different conclusion: he thought that the botryoidal tissue arose not fro the swelling of the cells forming the wall of a vessel, but from the end-to-e alignment of connective-tissue corpuscles which then formed an intracellul channel within their substance. These later connected with the vascu system and with the sinuses. Bourne believed that vaso-fibrous tissue dev loped from the botryoidal tissue by division of its cells, loss of their nuclei i the blood-stream, and then either total occlusion of the lumen or a final tra formation into thin-walled capillaries. Neither of these two workers postulat any function for the botryoidal tissue, and they did not discuss the nature the pigmented granules; Lankester does, however, point out a physiologi similarity between these granules and those forming the chloragogen of t earthworm.

A further suggestion as to the origin of the botryoidal tissue was due Cuénot (1891), who held that it arose from blood-vessels, and took the form blindly ending diverticula. The botryoidal cells he considered to be of am bocytic origin, accumulating pigment and then bulging outwards through t vessel wall. Cuénot thought that the vaso-fibrous tissue was not in comm nication with the botryoidal tissue, that it arose from connective-tissue ce and that it was blind at both its ends.

The next major contribution to this problem was that of Goodrich (189 he was concerned primarily with the vascular system of *Hirudo*, and he show conclusively that the sinuses, blood-vessels, and the botryoidal tissue were in communication with each other. The latter tissue actually occurs in a la in the connective tissue of the body of the leech, between the body-w musculature and the gut. Goodrich showed that it 'connects on the outs e 'intermediate capillary plexus', which is of sinus origin, and on the inner with sinuses passing to the 'intermediate layer'. He also made two further ts of interest, the first of which was that there are valves present in the ular system at the junction of the latero-lateral and the latero-dorsal els with the main lateral trunk, so that there is a true continuous circula- of the haemolymph into the main lateral vessel from these branches. This ns to show that the trend of flow through the botryoidal vessels is uni- ctional, and passes from the periphery to the deeper layers of the body. second interesting feature was that the contractile system (i.e. the blood- els) and the non-contractile (the sinuses) are in direct contact by means of -walled capillaries, and that the botryoidal tissue forms an alternative or by-pass through which the haemolymph may pass on its return from more peripheral parts of the body to the lateral vessels, which are the main ecting channels.

n 1905 Speiss studied the botryoidal tissue, or as he called it 'the peritoneal of the coelomic epithelium', from a more functional aspect. He based his clusions on the results of experiments in which dyes were injected into the odermis and gut of leeches. If the colouring agent was injected into former position, it rapidly appeared in the botryoidal cells, whereas if the ction was into the gut, then these cells did not show any trace of the dye, n after many months. These studies led him to the conclusion that the yoidal cells are excretory and are analogous to the chlorogogen cells of hworms, or to the liver-cells of higher vertebrates. In a second paper eiss, 1905*b*) he expressed the opinion that their pigment was in fact a pigment, though this opinion seems to have been based entirely on the lts of tests carried out on the water in which the animals had been kept, not on any *in situ* histochemistry. Abeloos (1925) also thought that this ue was excretory, in the sense that it must be considered as a kidney of umulation. He believed that it served a function which was divided in the nchobdellid leeches between the coelomic epithelial cells (the 'acid' cells Kowalevsky 1897), and the large cells which contain the yellow pigmented eres. This did not seem to be acceptable to Cuénot, who published a her paper in 1931 expressing his own ideas of the homologies of the various ds of pigmented tissue in leeches. He based his conclusions on the phago- c behaviour of the cells to injections of ammonium carminate and indigo phate. In *Hirudo* the botryoidal cells take up the former dye, so resembling coelomic epithelial cells of the rhynchobdellids, whilst the indigo sulphate ccumulated by the vaso-fibrous tissue of *Hirudo* and by the large pig- nted cells of the other group of leeches.

MATERIAL AND METHODS

he animals used in this study were obtained from a firm of laboratory pliers and kept in glass aquarium tanks until required. Most of the work done with mature adults, but for certain special purposes young in- duals not more than 2 cm in length were used.

Living cells were studied in preparations dissected out in saline a mounted unstained in the same liquid for examination by positive and neg tive phase-contrast and interference microscopy. For vital dye studies, t tissue was stained with neutral red, dahlia violet, methylene blue, and Jan green B; the dyes were dissolved at a concentration of 1% in distilled wat and then diluted with 0·75% saline to a final concentration of 0·01%. T tissues were immersed in the dye for periods varying from 10 to 20 min. befo being finally mounted in saline.

Some extractions of pigment were made for *extra situm* histochemi studies; this was done by dissecting out the connective tissue containing t botryoidal tissue and then dissolving the pigment from this in 0·1 N NaOH in glacial acetic acid.

Animals were also fixed, sectioned, and subjected to standard histochemi tests as listed in the appendix to the paper.

The structure of the botryoidal tissue

When spreads of connective tissue from the region of the body immediate dorsal to the gut are examined, the brown botryoidal tissue is very conspicuou appearing as vessels with many clusters of pigmented cells forming divertic or caeca. These cells are found along the length of the botryoidal tissue a they give it a characteristic bulbous appearance in such preparations (fig. In surface view the cells appear to be oval, about 15 μ along their shorte dimension and often over 35 μ long; though their basic shape seems to ovoid, the cells are very often pressed together so closely that some distorti of shape results.

The whole cytoplasm of the cells is full of spherical pigmented granul approximately 1 μ or less in diameter. Often a clear space is visible, usual about 5 μ in length, which represents the position of the nucleus.

In spreads of connective tissue taken from mature leeches, the appearan of the botryoidal tissue is slightly misleading; the cells are so full of pigme granules and are so swollen that they appear to be bulging outwards from t wall of the botryoidal vessel (fig. 1). If, however, a spread is made from t connective tissue which occurs at the point where the botryoidal tissue term nates, then places may very often be seen in the spread where the botryoid cells are smaller and often separated from each other by considerable distanc It is then clearly seen that these botryoidal cells are, in fact, the cells which for the wall of a vessel, and that when they begin to accumulate pigment granul they bulge inwards into the lumen of the vessel (fig. 2). It is sometimes po sible to see a cell which has begun to bulge inwards but has not yet accumulat more than a few pigment granules, which tend to occur in the basal region the cell. It is presumed, therefore, that the appearance of cells swelling ou wards from the vessel is a secondary one, and is a consequence of the specializ tion of the cell for the accumulation of pigment granules. A thin protoplasm layer representing the original wall of the vessel is found to occur on t outer surface of all the botryoidal cells. It is very difficult to see in livi

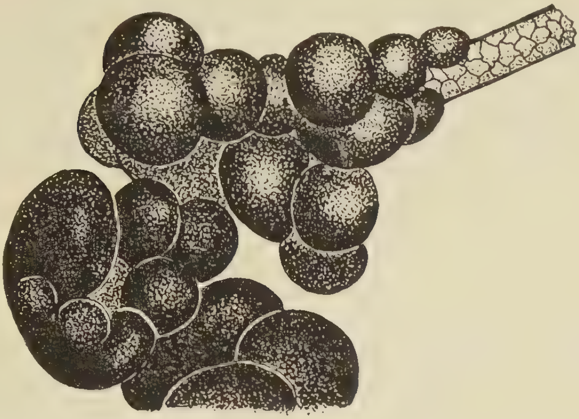

FIG. 1. Botryoidal tissue of *Hirudo* seen in a preparation of connective tissue which was spread on a slide and not compressed. The swelling of the cells and origin from a thin-walled capillary can be seen.

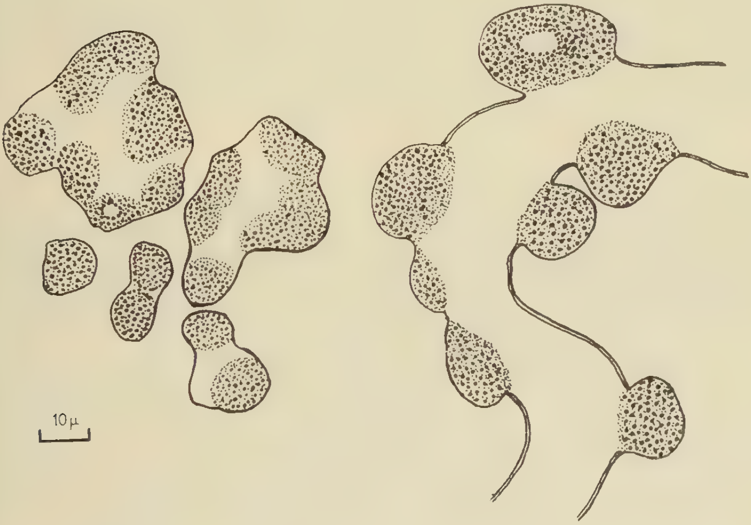

FIG. 2. Botryoidal tissue as seen in section. The cells in the transverse section are still bulging inwards into the lumen of the vessel, whereas those in the longitudinal section on the right have begun to swell outwards.

eparations, but it was found that in sections stained with the pyronin/ethyl green technique (Jordan and Baker, 1955), the vessel wall coloured tensely with the pyronin and could be clearly seen on the outer surface of the tryoidal cells.

In the spreads of connective tissue from young leeches, the number of the tryoidal cells seemed to be less than in the old leech, and many of the cells

seemed to be smaller. The principal appearance was of cells bulging into t
lumen of a vessel, but there were patches of tissue where the cells were mu
more numerous and seemed to be as dense as in the tissue of much old
leeches. It was often noticed that a section of thin-walled capillary would sh
a patch of well-developed botryoidal cells, and perhaps 100 μ farther alo
the same vessel would revert to thin-walled capillary again; many sm
diverticula or caeca whose cells were in the process of becoming botryoi
were noticed in the tissue of these small animals.

In an attempt to discover whether there was any correlation between t
age of the leech and the amount of botryoidal tissue present, two separ
experiments were tried. The first simply consisted of removing the skin of t
dorsal surface and the immediately underlying body musculature from bot
young and an old leech; a low-power dissecting microscope was used to che
that only skin and muscle was removed. The botryoidal tissue was then app
rent, as shown in figs. 3, A and 3, B. Fig. 3, A represents the old leech, whi
fig. 3, B is of a young animal, 2 cm long. In the older animal it was clear t
the whole of the dorsal surface of the body immediately below the bo
musculature was occupied by the botryoidal tissue, which was reddish wh
seen in the mass. The younger animal had much less pigment and many ar
of the body could be seen to be without pigmented tissue; in this leech t
pigment was much darker than in the older specimen. This result seems
indicate that there is a definite increase in the amount of botryoidal tissue
the animal grows older.

The other experiment was carried out on unstained transverse sections
both young and old leeches. In each case they were studied with a 4-mm obj
tive and with an eyepiece which was fitted with a graticule divided into qu
rants. The number of botryoidal cells visible in each quadrant of the field
view was counted in each of 10 microscopic fields taken along the part of
body in the region dorsal to the gut and below the body musculature;
results are expressed in the form of a histogram (fig. 4), in which the numbe
quadrants is plotted against the number of cells per quadrant. It is seen fr
the diagram that there is a very significant increase in the amount of botryo
tissue in the older leech. A further point which is obvious from a study of
histogram is that there is no major change in the density of the botryoi
tissue, once a certain value has been reached. It must be emphasized here t
these results are taken from two arbitrarily chosen stages in the life-hist
and that there are no observations on the very young leeches which might
supposed to show the early stages of the process of botryoidal tissue format
it would prove very interesting to repeat these measurements on much sma
leeches, preferably those which have not yet had their first meal of bloo

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FIG. 3 (plate). A, a photograph of an old specimen of *Hirudo* with the skin and body mu
lature of the dorsal surface removed. Note that the whole surface is covered with pigme
tissue. B, a similar preparation to that shown in A, but of a young leech; note that the
considerably less pigmented tissue, but that it is darker.

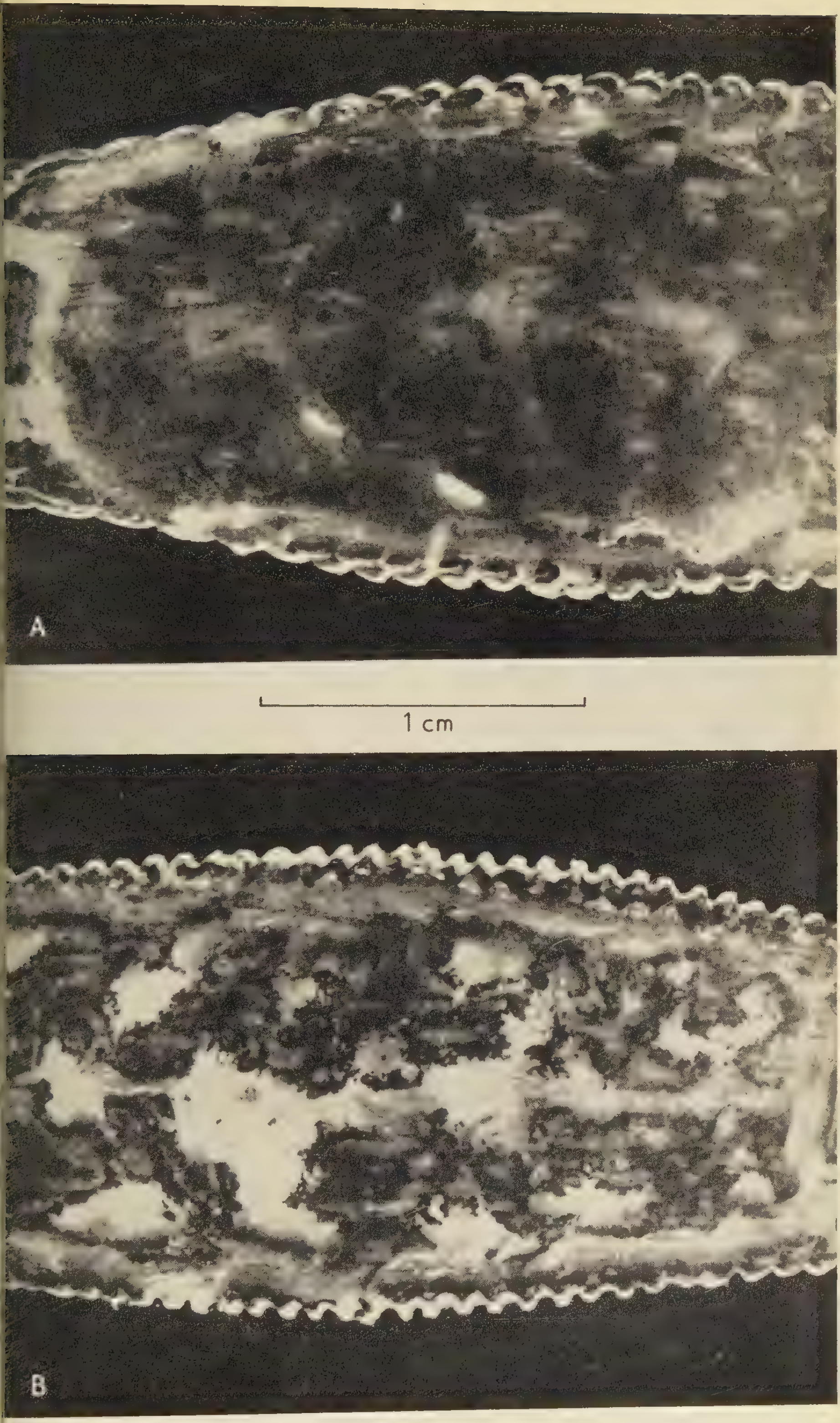

Fig. 3

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t thus seems as if one may conclude that the botryoidal tissue of *Hirudo* s increase in amount as the animal ages, and that it reaches a certain density the connective tissue. If, as is supposed, the botryoidal cells arise from nges in the epithelial cells of vessels or sinuses, then this is exactly what uld be expected. The increase in total amount represents the spread of the nge to other cells of the vessel walls at different places in the connective ue.

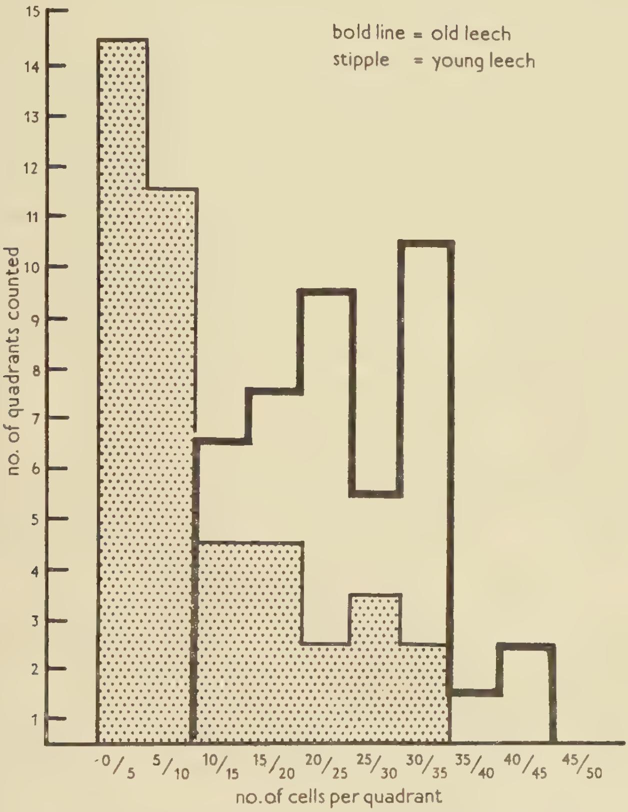

FIG. 4. A histogram showing the relative amounts of botryoidal tissue in equal areas of section of young and old leeches.

e histochemistry of the pigment granules

Details of the histochemical tests applied to the pigment granules are mmarized in the table given as an appendix to the paper. The pigment was und to be very soluble in 0·1 N NaOH and in glacial acetic acid, and it was colourized in a few hours by a 10% solution of hydrogen peroxide. This, wever, is considered as a bleaching of the pigment rather than as its solution. ter the bleaching of the pigment by hydrogen peroxide, the granules were ll seen to be present in the botryoidal cells; as they were now colourless, ey were subjected to further tests intended to reveal their nature. From the appendix it can be seen that they gave a positive reaction to the protein tes such as the Sakaguchi reaction (Baker, 1944), the Hg/nitrite test (Baker, 195 and the coupled tetrazonium reaction (Danielli, 1947; Pearse 1954). It is th concluded that the granules contain a protein 'framework' for the pigmen Here one notes a similarity to the pigmented spheres in the large pigment c of the rhynchobdellid leech *Glossiphonia complanata* (Bradbury, 1957). N reactions were obtained from the tests for lipids. The positive result given b the performic acid / Schiff test is thought to be due to some unsaturation the protein component of the pigmented spheres.

In view of the results obtained in the histochemical study of the leech *G. con planata* it was thought possible that in *Hirudo* either a bile pigment or iron-containing compound might be responsible for the colour of the pigmen Two reasonably specific tests, those of Gmelin and Fouchet, are availab for testing for the presence of a bile pigment, whilst the acid ferrocyani test of Perls, carried out both with and without a pretreatment with hydrog peroxide, is used for the detection of iron. In the case of mature, we pigmented botryoidal tissue, there was a negative reaction to both Fouchet and Gmelin's tests, though in those cells which had not yet accumulated the full complement of pigment granules and which were still small enough bulge inwards into the lumen of the vessel, there was a positive reaction the Fouchet test.

With both the old and the young *Hirudo* there was a definite positi reaction to Perls's test for iron, both in the pigmented granules and also in th general cytoplasm of the botryoidal cells. In the young leech many spheric granules, about 2 μ or more in diameter, and intensely positive to the test f iron, were found in the botryoidal cells; these granules may perhaps represe pigment granules which have aggregated under the conditions of the test. I the old leech it was noted that many of the pigment granules, particular those towards the periphery of the cells, were uncoloured by the test, an appeared in the section in their natural colour. When the sections were treate with 100-volume hydrogen peroxide for half an hour before applying the tes there was found to be little change in the sections of the young leech; in th botryoidal cells of the old animal, however, there was now found an inten positive reaction from each granule. It thus seems that both young and o leeches possess iron in the pigment granules of their botryoidal tissue; in th young leech and in some of the granules (presumably those most recent formed) of the botryoidal cells of the old leech, the iron seems to be prese in the ionic state. In the majority of the botryoidal cell granules of the old animal the iron is obviously bound up into more intimate contact with th protein of the granule framework and so is rather unreactive and is said to b masked.

It is interesting to note in passing that spherical granules 1 μ in diamete which gave a strong positive reaction to the Perls test (without hydroge peroxide treatment), were also found in the intestinal epithelium of the youn leech; they were concentrated around the nuclei of the cells and often seeme be aggregated into chains or larger clusters. Here again is a condition which paralleled in the rhynchobdellid leech (Bradbury, 1955). Another place ere similar granules were found in *Hirudo* was in the cells forming the wall some of the coelomic channels. It was also noticed that after the unmasking atment the coagulated fluid in these channels was weakly positive to the t, probably on account of the haemoglobin contained as the respiratory gment in the haemolymph of this leech.

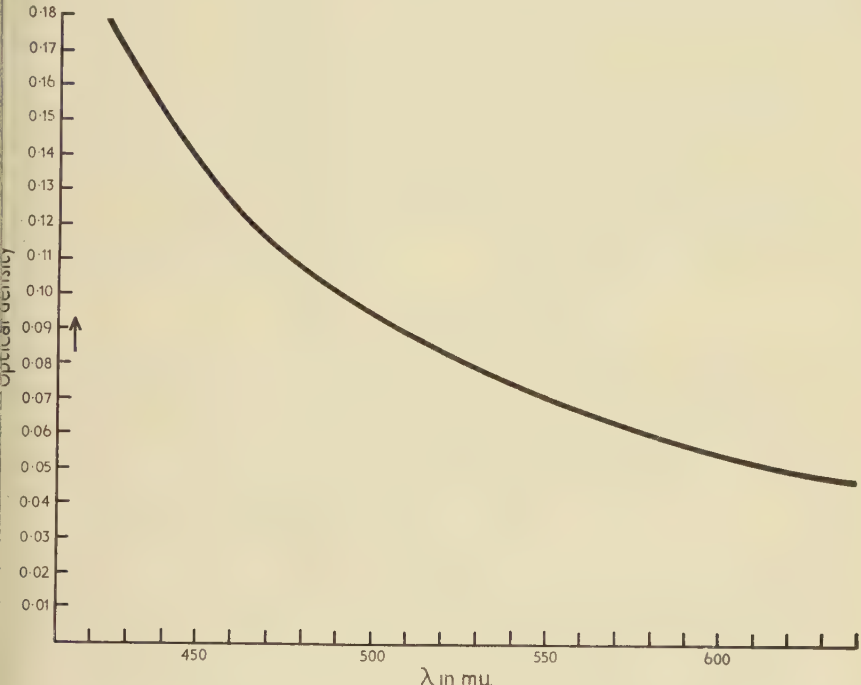

FIG. 5. Absorption curve of the pigment extracted from *Hirudo*.

When the solubility of the pigment in alkali and acetic acid was discovered, fficient solution was extracted from one leech to enable some of the tests to repeated, and also to permit measurement of the absorption in the visible gion of the spectrum; the resulting absorption curve is shown in fig. 5. is apparent that there are no obvious absorption bands, so that no definite nclusions can be drawn from this test. Attempts to obtain a positive Gmelin Fouchet reaction on this extract were not successful.

The ground-cytoplasm of the botryoidal cells was found to contain some S-positive material which resisted saliva digestion and was not metachro-atic; it thus seems to belong to the class of substances termed neutral ucopolysaccharides. There was also a diffuse lipid reaction in the ground toplasm when Sudan black was used after the phenol unmasking technique devised by Ciaccio (1926). A few very small spherical granules were notice which gave a positive reaction to the acid haematein test.

The vaso-fibrous tissue

In most preparations of connective tissue from *Hirudo* it is usually possib to see, in addition to the botryoidal tissue, a second pigmented tissue, whic appears much more fibrous in character. This is the vaso-fibrous tissue, fir

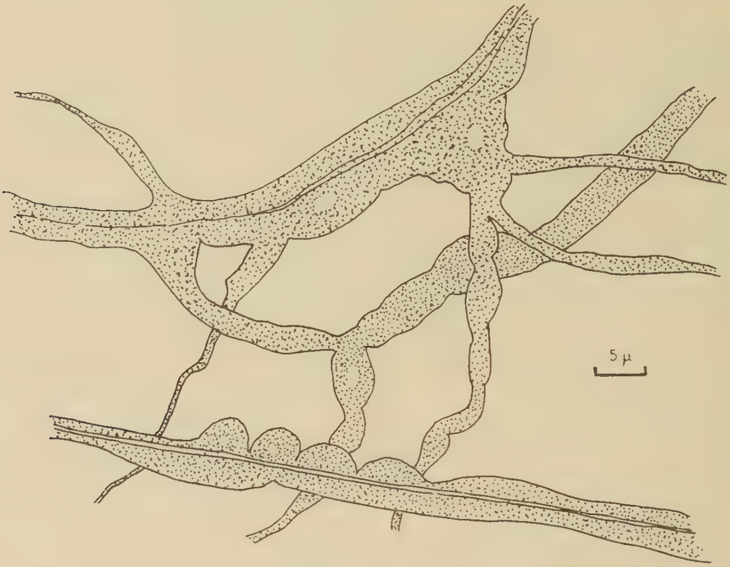

FIG. 6. Drawings of the appearance of the vaso-fibrous tissue in living preparations. Note the positions of the nuclei, and the indication of the position of the lumen. The strand of tissue in the lower portion of the figure shows the outward swelling of the cells mentioned in the text.

described and named by Lankester in 1880. It ramifies very extensive throughout the ground-substance of the connective tissue, and has the gener appearance shown in fig. 6; although the diameter of the 'fibres' is variable, is usually between 3 and 5 μ.

Cuénot was of the opinion that this tissue formed a blindly ending syste with no communication with either the blood-vessels or the botryoidal tissu and that it had a regular layer of pigment granules surrounding a central co of cytoplasm. Other authors such as Lankester (1880) and Bourne (188 have definitely stated, however, that this tissue is in connexion with t botryoidal vessels on one hand, and with the capillaries on the other.

From observations during the course of the present study, carried out both sections and spreads of living connective tissue, it appears that La kester's view is indeed the correct one, the vaso-fibrous tissue seeming alwa to have a lumen, even if it is quite small. The pigment granules occur in t cells which form the wall of this structure, and it is this which probably misl enot into thinking that they formed a regular layer around the periphery central core of protoplasm. These pigment granules are very small (often than 1 μ in diameter); when they are seen in the living tissue they are en, thus contrasting strongly with the pigment granules of the botryoidal ue.

n living material it is very often possible to see the transition from thin-lled capillary to typical vaso-fibrous tissue, as well as places where the vaso-rous tissue enters a botryoidal vessel. From a study of sections it seems that channels of the vaso-fibrous tissue are filled with a coagulated fluid which embled the haemolymph in its staining properties.

Though the typical appearance of this tissue is that of a pigmented fibre of iform diameter with a central lumen, occasionally some parts of it have the pearance of a vessel with the cells of the wall swollen outwards by the cumulation of pigment granules, as shown in the lower part of fig. 6; this ggests that the process of formation of this tissue from a thin-walled capil-y occurs in a very similar way to that of the botryoidal tissue, but that the gree of swelling is much less.

stochemistry of the vaso-fibrous pigment

During the course of the histochemical study of the botryoidal tissue, it was ssible to record the results of the same tests on the pigment of the vaso-rous tissue, as this was almost invariably present in the same sections. The tails of the tests are presented in the appendix of the paper, but some points em to call for comment.

It was found that the cytoplasm of the vaso-fibrous cells, inside the pigment anules, showed many small spherical granules which coloured very strongly th the vital dye dahlia violet; these may represent the lipochondria of the lls. It was thought that they might contain lipids, but it proved very difficult distinguish them, as the contents of the lumen also coloured slightly with e colouring agents for lipids. In places, however, where the plane of section as tangential to the vaso-fibrous strand, it was possible to observe that these all granules did in fact give a positive reaction to the acid-haematein tech-que, and so may be supposed to contain phospholipid.

The pigment granules were found to resemble those of the botryoidal sue in possessing a protein 'framework', but the pigment appeared to be fferent. The Fouchet test was always strongly positive, but the Gmelin st was not. It is known, however, that these tests are capricious (Pearse, 54) and no significance is attached to this negative result. The test for iron as invariably negative, even after the unmasking treatment had been applied. thus seems possible to conclude that the pigment of the vaso-fibrous tissue free from iron, and probably resembles a vertebrate bile pigment.

DISCUSSION

Many different views have been proposed to explain the origin and nature of e botryoidal and vaso-fibrous tissues of the leech *Hirudo medicinalis*, with the result that there is much confusion; the chemical composition and fun tions of these tissues appear to have been rather neglected. As a result of t present observations it seems that the view originally put forward by Gratio (1862) and amplified and clarified by Lankester (1880) should be accepte namely, that the botryoidal tissue represents the swollen cells of vessel wall It seems likely that this is also true of the vaso-fibrous tissue. Both these tissu form independent systems which communicate freely with each other and all with the sinus and vascular systems. As a result of Goodrich's careful work also appears that these systems are not the sole means of communicati between the contractile and non-contractile elements of the circulato system. If this view of the nature of the botryoidal and vaso-fibrous tissues accepted, then it follows that Cuénot (1931) was correct in assuming a corr spondence with the typical swollen coelomic epithelial cells of the rhy chobdellid leech.

From the results of histochemical studies reported in this paper, it seem that the green pigment of the vaso-fibrous tissue is a tetra-pyrrol, whic closely resembles the bile pigments of vertebrates; the pigment in the bo ryoidal tissue, though it may contain some bile pigment is chiefly composed of bound iron compound. The presence of these two pigments may be correlate with the blood diet of this animal. It has already been suggested (Bradbur 1957) that these products would result from the breakdown of the haem pi ment which is part of the ingested blood. Although we have no direct eviden of the site of breakdown, it is interesting to speculate a little on this problem Since granules containing iron have been noticed in the intestinal epitheli cells, it may well be that the primary breakdown of the blood pigment, wit the consequent splitting-off of the iron, takes place in the cytoplasm of th intestinal epithelium. The remaining proto-porphyrin and the inorganic ir might pass in the haemolymph to the botryoidal and vaso-fibrous tissue. possible alternative view is that the actual metabolism of the haemoglobi takes place in the cytoplasm of the cells of one of these tissues. As the cyto plasm of the vaso-fibrous tissue seems to contain many more inclusions tha that of the botryoidal tissue (the pigment granules excepted), it may be that is more active and represents the site of breakdown of the proto-porphyrin the products of which then accumulate in the cells and form the green pigmen granules. From the lack of cytoplasmic inclusions in the botryoidal cells, may be doubted whether these cells are metabolically very active; it seem more likely that they are acting as storage centres. The observed increase i the amount of botryoidal tissue in the older animals seems to accord with thi and lends support to the idea of Abeloos (1925) that these cells represent kidney of accumulation, where the by-products of a metabolism occurrin elsewhere in the body of the animal are stored. When this situation is com pared with that existing in *G. complanata* (Bradbury, 1955; 1957), it seems tha the vaso-fibrous and botryoidal tissues of *Hirudo* may be considered to b functionally equivalent in this respect to the adipose cell of *Glossiphonia*. Th specialized part of the cytoplasm of this cell which accumulates the larg gmented spheres would represent the vaso-fibrous tissues, whilst the general toplasm of the adipose cell is the functional iron-storage centre, just as the tryoidal tissue is in the other leech. It is known that there are no adipose ls in the connective tissue of *Hirudo* or the other gnathobdellid leeches, and e functions of this most important cell-type appear to have been allocated to her parts of the body; the fat is stored in the cells of the crop epithelium d in the fibrocytes (Bradbury, 1958), whilst the accumulation of the meta-lic waste products have become associated with the cells forming the emolymph vessels. It may also be that the chief metabolic activities of the ipose cell cytoplasm have become associated with the cytoplasm of the cells ming the vaso-fibrous tissue.

The evidence seems to suggest that in *Glossiphonia* the bile-like pigment is cumulated in the adipose cells and that the iron is stored there before its entual excretion. In *Hirudo* the iron seems to be stored throughout the life the animal in the botryoidal tissue, in a manner similar to that in which iron cumulates throughout life in the gut cells of the reduviid bug *Rhodnius olixus* (Wigglesworth, 1943). At the moment, this difference between these o species of leech remains unexplained, though further experiments may lp us to understand it. *Glossiphonia* is not known to have any respiratory gment in the blood or coelomic fluid, whereas *Hirudo* is reported to possess emoglobin dissolved in the plasma of the haemolymph. The iron may be red in the botryoidal tissue, rather than excreted, in order to ensure that ere is always sufficient to provide for synthesis of the animal's own respira-y pigment.

I have great pleasure in again acknowledging my debt to Dr. J. R. Baker, R.S., for his most helpful encouragement and advice at all stages of the rk. My thanks are also due to Professor Sir Alister Hardy, F.R.S., for oviding facilities in his department. Financial assistance from a Senior ulme Scholarship of Brasenose College, Oxford, and from the Department Scientific and Industrial Research, is gratefully acknowledged. I am grate-to Miss Christine Court for help in preparing the figures for publication.

APPENDIX

A summary of the histochemistry of the botryoidal and vaso-fibrous tissue of t *leech* Hirudo

Test	Reference	*Results*		
		Cytoplasm of botryoidal cell	*Botryoidal pigment*	*Vaso-fibrou pigment*
colour	—	—	brown	greenish
solubility H_2O	—	—	O	O
alcohol	—	—	O	O
carbon disulphide	—	—	O	O
chloroform	—	—	O	O
ether	—	—	O	O
glacial acetic acid	—	—	+++	+++
0·1 N NaOH	—	—	+++	+++
benzene	—	—	O	O
acetone	—	—	O	O
Methylene blue extinction	Pearse, 1954	pH7	pH 3	—
H_2O_2 bleach	—	—	+++ after several hours	+++ after several hours
Mann-Kopsch	Weigl, 1910	weak general impregnation	++	++
Aoyama	Aoyama, 1929	some granules +++	some + some O	+granules near cen of tissue
Sakaguchi	Baker, 1947	+	+++	+++
Hg/nitrite	Baker, 1956	++	+	+
coupled tetrazonium	Pearse, 1954	+++	pigment appears in natural colour	pigment appears i natural colour
C.T. and benzoylation	,,	O	pigment granules are swollen and yellow	pigment granules are swoll and yello
C.T. and D.N.F.B.	,,	+++	pigment appears in natural colour	pigment appears i natural c lour
C.T. and performic acid	,,	+++	pigment granules swollen and red	pigment granules swollen a red
PAS	,,	++	O	O
PAS and saliva	,,	++	O	O
PAS control	,,	O	O	O

APPENDIX (*cont.*)

Test	Reference	Results *Cytoplasm of botryoidal cell*	 *Botryoidal pigment*	 *Vaso-fibrous pigment*
line blue	Pearse, 1954	orthochromatic	O	O
h black	Baker, 1944; 1949	*few* +++ granules	O	O
h IV	Herxheimer, 1901	*few* +++ granules	O	O
after Cajal	Ciaccio, 1926	++ diffuse	O	O
after Da Fano	,,	O	O	O
after phenol	,,	++ diffuse	O	O
A.S.	Pearse, 1951	O	+	+
blue	Cain, 1947	O	O	O
	Baker, 1946	*few* granules +++	O	granules inside pigment layer +++; pigment O, lumen contents ++
control	Baker, 1946	O	O	O
g	Jordan and Baker, 1955	O wall coloured +++ pyronin	O	O
g control	Bradbury, 1956	O wall coloured +++	O	O
eration	—	O	+++	O
	Gomori, 1952	young ++ diffuse;	+++	O
	,,	old O	++ some	O
after H_2O_2	,,	young ++ diffuse;	+++	O
		old ++ diffuse	+++	O
lin	Tiedmann and Gmelin, 1826	O	O	O
chet	Quoted by Cole, 1955	O	+ sometimes	+++
ia violet	—	O	O	granules inside pigment layer +++

+++ = strong reaction; ++ = medium reaction; + = weak reaction; — = no observation; O = negative reaction.

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he Use of Teased Preparations and Frozen Sections in uantitative Studies of Mammalian Peripheral Nerve

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With one plate (fig. 4)

SUMMARY

hen teased preparations of peripheral nerve are examined, the highly refractile lin sheath acts as a convex lens and affects estimates of axon diameter and sheath kness. The construction of ray diagrams for different axon-sheath ratios indicates the degree of magnification varies with this ratio. Models and experiments in h teased fibres were immersed in fluids of different refractive index show that nification also varies with the relative refractive indices of the sheath and the ersion medium. Fibres from the nervus gastrocnemius medialis (NGM) of the it were teased in Ringer's solution and measurements made with a polarizing roscope. A regression line for 2 × apparent sheath thickness on external diameter calculated.

rozen sections (Williams, 1959) of the NGM were prepared, and from these vidual fibres were tipped over on to their sides by micromanipulation. A magnifin effect similar to that existing in teased fibres was thus introduced and a compari of their apparent axon diameters was made valid. An analysis of co-variance ved no significant difference in variance, slope, or elevation. It was concluded these frozen sections provided a satisfactory basis for an estimate of magnifica, and that estimates of size relationships made on these frozen sections reflected natural state as closely as estimates made on isolated teased fibres and corrected magnification.

regression line was derived for 2 × myelin sheath thickness on external diameter n intact frozen sections. This line was compared with the regression line for teased es and percentage magnification of the axon plotted against external diameter. graph indicated that this percentage magnification decreased with fibre diameter n over 40% to under 20%.

INTRODUCTION

N attempts to produce quantitative data representing nearly the natural state, observations have been made on nerve-fibres freshly teased in ger's solution.

ince the classic studies of de Renyi (1929), teased preparations have been d in the study of paranodal morphology by Lubinska and Łewkaszewska 56) and Higgs (1958); in an investigation of sheath birefringence by mitt and Bear (1939) and Taylor (1942); as a basis for assessing the effects processing by Sanders (1948); and during physiological investigations by ny workers, e.g. Müller (1958).

t has been pointed out (Ashley, 1958; Williams and Wendell-Smith, 1958) t when teased preparations of peripheral nerve are examined, the optical

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properties of the myelin sheath and the immersion medium produce appare changes in size relationships. At the uncomplicated internode the axon subjected to the magnifying effect of a cylindrical convex lens of myeli this effect being modified by the properties of the surrounding immersi medium. Thus the refractive indices at two interfaces are of importanc myelin / immersion medium, and immersion medium / air. The present pap presents an analysis and assessment of this situation and a comparison teased preparations with frozen sections.

MATERIALS AND METHODS

Determination of refractive indices. The refractive indices of the immersi media used were determined with an Abbe refractometer. Estimations of th appropriate refractive index of peripheral nerve myelin were made by th contrast-reversal method (Barer and Joseph, 1955).

Construction of ray diagrams. By the use of these refractive indices, r diagrams were constructed for models fibres having three different axon / shea ratios.

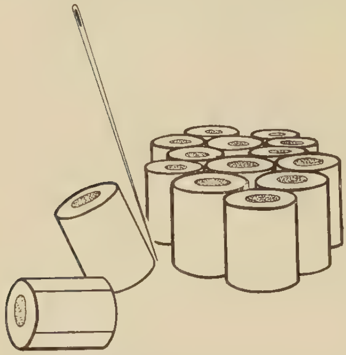


FIG. 1. Micromanipulation of individual fibres of a frozen section to put them in position for viewing from the side, thus causing magnification of the axon.

Experimental demonstration of magnific tion. Glass capillary tubing was immerse in fluids of different refractive indices an photographed in side view to represent teased fibre and end-on to represent a tran verse section.

Teased nerve-fibres were immersed fluids of different refractive indices an photographed.

Teased fibres in Ringer's solution. Fibr carefully teased in Ringer's solution we examined by the polarizing microscope an photographed. Measurements of extern (total) diameter and apparent axon diamet were subsequently made on photographs at final magnifications of × 1,0 and × 2,000. Measurements were made at 5 places in each internode and th mean values recorded. The maximum error of the measuring technique w ±4%.

A scatter diagram of external diameter against apparent sheath thickne for 92 fibres was prepared and a regression line and standard error of estima were calculated.

Frozen sections and teased preparations. For comparison with the tease fibres, frozen sections were prepared by the technique of Williams (1959 Microdissectors (Barer and Saunders-Singer, 1951) were used to detach fibr from sections and tip them over on to their sides (fig. 1). The tipped-ov fibres were then photographed on their sides and similar measurements mad measurements from 68 tipped-over fibres were compared with those teased fibres.

nilar measurements were made on intact frozen sections and a scatter am for 204 fibres was prepared. A regression line and standard error timate were calculated.

ally, the data derived from teased preparations were compared with from intact frozen sections and a magnification curve plotted.

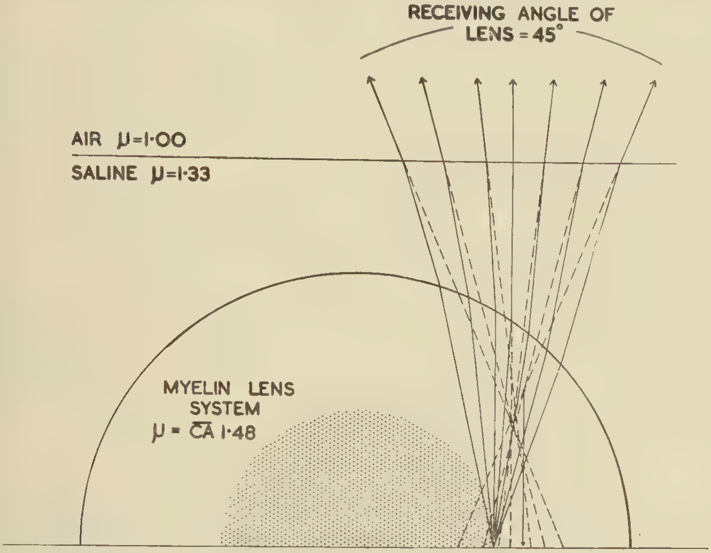


FIG. 2. Scale-diagram of a nerve-fibre having an axon / 2 × sheath ratio of 1/1, immersed in saline. The production of multiple virtual images of the axon margin is illustrated.

RESULTS

ie first scale-diagram (fig. 2) indicates the principles involved. A light rom the axon edge is refracted first at the surface of the sheath and then surface of the immersion medium. When the emergent rays are plotted virtual images are found lying on a caustic curve. This multiplicity ages accounts in part for the rather poorly defined axon margin seen in d fibres.

. 3 illustrates the position of caustic curves for two other axon / sheath . The different relative positions of the caustic curves indicate that ification varies with this ratio.

pillary tubing viewed side-on in fluids of varying refractive index exhibits variations in apparent axon / sheath ratios (fig. 4, A, B, C). Similarly, ve-fibre immersed in fluids of differing refractive indices shows variation parent axon diameter and apparent sheath thickness (fig. 4, D, E). In ion, nerve-fibres were teased in saline and photographed during the ion of glycerine (fig. 4, J). On the left the appearance is comparable with

that of fig. 4, D; the glycerine has not reached the fibre at this point and
birefringent myelin sheath is apparent. On the right the myelin sheat
scarcely visible owing to the high refractive index of the glycerine, which
reached it. Here the relatively weak birefringence of the axon is manifest.
of interest to compare the apparent size of the axon in these two situati

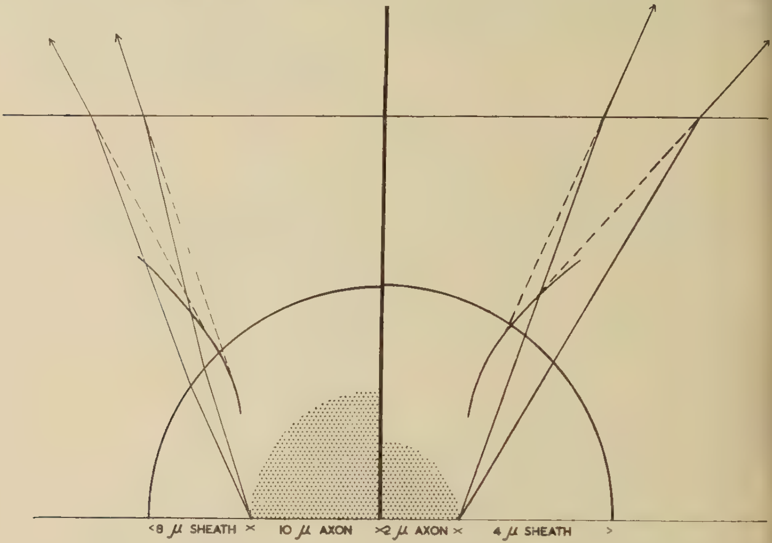


FIG. 3. Scale-diagram of two nerve-fibres of different axon / sheath ratios, showing the different positions of the caustic curves of virtual images.

The appearance of a fibre carefully teased in Ringer's solution is seen fig. 4, I. Fig. 5, A is a scatter diagram of apparent sheath thickness agai external (total) diameter for such fibres. A positive linear correlation evident. The calculated regression line and relevant statistics are given fig. 5, B.

To measure magnification a base line was necessary and it seemed lik that frozen sections of fresh peripheral nerve would provide this. It necessary therefore to determine whether the technique of sectioning resul

---

FIG. 4 (plate). A, B, and C are photographs of lateral and end-on views of the same capil tube in A, air; B, saline; C, glycerine. The end-on dimensions remain the same in the t media; when viewed from the side the external diameter remains the same but the appa internal diameter decreases with increasing refractive index of the immersion medium.

D and E are polarized light photomicrographs of the same teased fibre in two fluid different refractive index. Note the relative sizes of axon and sheath in the two cases.

F, G, and H are polarized light photomicrographs of individual fibres detached from fr sections and viewed side-on. Note the preservation of cylindrical form.

I, a polarized light photograph of a myelinated nerve-fibre teased in Ringer's solu Note the shearing defects of Schmidt–Lanterman type.

J, a single nerve-fibre teased in saline and photographed by polarized light, du-ing addition of glycerine. For further description see text.

In A–J the bar indicates 10 μ.

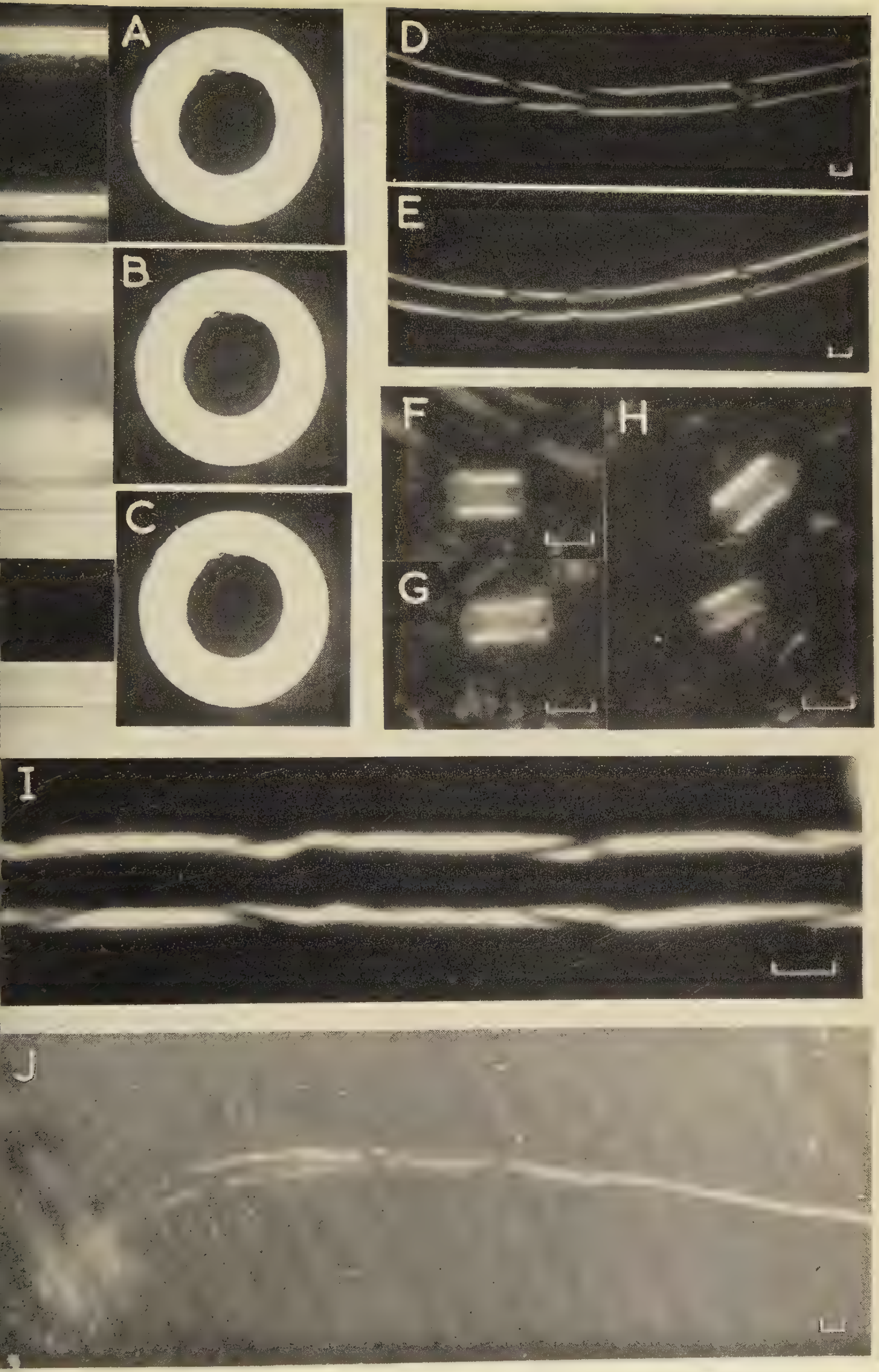
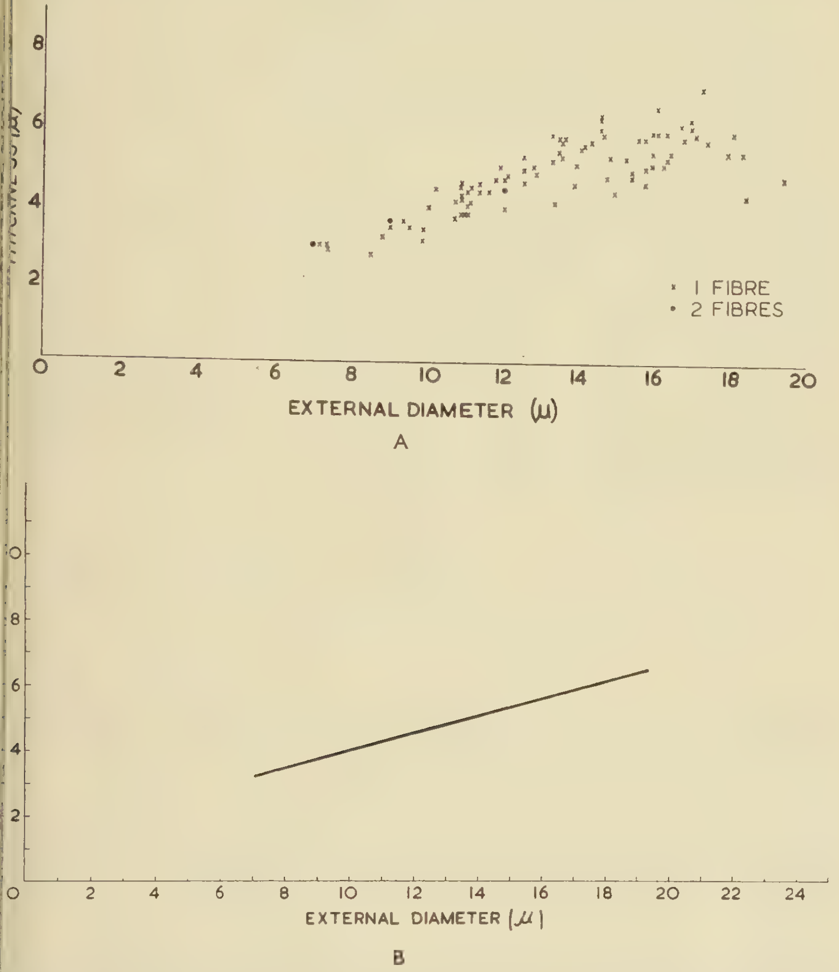


FIG. 4

C. P. WENDELL-SMITH *and* P. L. WILLIAMS

e relationships which differed significantly from those of teased fibres. fact of magnification having been demonstrated, it is clear that fibres d end-on (i.e. in section) should not be compared directly with teased viewed from the side. If, however, sectioned fibres could be viewed from



. A, scatter diagram of 2 × myelin sheath thickness on external diameter for 92 teased of the NGM of the rabbit, teased in normal saline. B, calculated regression line for data fig. 5, A. Correlation coefficient 0·8922; regression coefficient 0·2687; standard error of estimate of sheath thickness from external diameter 0·4047 μ.

ide (fig. 4, F, G, H), comparison would be acceptable. Regression lines for myelin sheath thickness on axon diameter for tipped-over fibres and d fibres were compared by an analysis of co-variance (Snedecor, 1956). total variance for each sample was partitioned and residual variance, ariance due to deviations from regression, estimated. The estimates of residual variance were compared by a variance ratio test. The res $F = 1\cdot 19, f_1 = 66, f_2 = 43$ lacks significance ($P > 0\cdot 25$), and thus pooling the two samples is permissible.

Sums of squares of deviations from regression were made in three ways

(1) by pooling the residual sums of squares, a measure of deviation fr *separate regression* lines;

(2) by pooling the sample sums of squares and products, a measure deviation from the *common regression* line;

(3) by pooling the original data, a measure of deviation from the *over regression* line.

Item (2) minus item (1) measures the difference between the regressi coefficients. Since the mean square of this difference is less than norm sampling variation as represented by item (1), we are justified in assumi that the slopes of the two regressions are the same.

Item (3) minus item (2) measures the sample difference in elevation. Sin the mean square of this does not differ significantly from that of item (($F = 1\cdot 76$, $f_1 = 1$, $f_2 = 111$: $0\cdot 25 > P > 0\cdot 10$), the population regressi lines may have similar positions. The overall conclusion is that the tw populations may have the same regression lines, any differences being t small to be detected by the sampling (table 1).

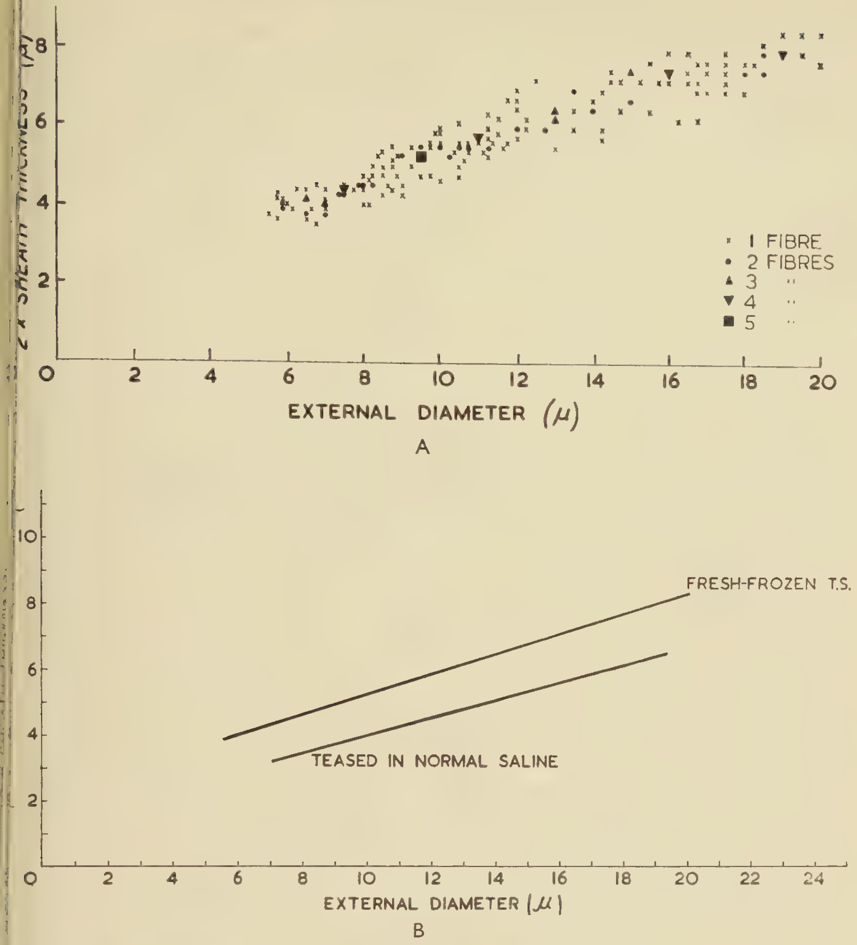
TABLE 1

Analysis of co-variance of data from tipped and teased nerve-fibres

Source of variation	Degrees of freedom	*Sums of squares and products*			Regression coefficient	*Deviations from regression*		
		Σx^2	Σxy	Σy^2		*d.f.*	$\Sigma y^2-(\Sigma xy)^2/\Sigma x^2$	*Mea squar*
tipped fibres	67	697·4	209·9	119·8	0·3008	66	56·6	0·857
teased fibres	44	194·6	61·7	50·4	0·3257	43	30·9	0·719
separate lines	..	..	..	..	..	109	87·5	0·802
differences in slope	..	..	..	..	..	1	0·1	0·1
common line	111	892·0	271·6	170·2	0·3046	110	87·4	0·795
differences in elevation	..	..	..	..	..	1	1·4	1·4
overall line	112	1032·3	293·6	172·3	0·2849	111	88·8	0·752

Accordingly we accept the intact frozen sections as a satisfactory base lin giving an estimate of unmagnified axon diameter. The data for 204 fibr from such sections are presented in fig. 6, A.

Fig. 6, B shows the regression lines for 2 M on external diameter for teas fibres and for frozen sections. An analysis of co-variance similar to that abo was precluded by the significant difference between residual varianc ($F = 1\cdot 75, f_1 = 202, f_2 = 90$: $P < 0\cdot 005$). Inspection of the regression lin and the corresponding standard errors suggest a marked difference in elev tion; accordingly, we consider the two lines represent different populati lines. These have been used to construct a magnification chart (fig. 7).



6. A, scatter diagram of 2 × sheath thickness on external diameter for 204 fibres from h frozen sections of the NGM of the rabbit. B, calculated regression lines for data from 5, A and 6, A. The statistics for the teased data are as in fig. 5. For the frozen sections: elation coefficient 0·9291; regression coefficient 0·3099; standard error of estimate of sheath thickness from external diameter 0·5287 μ.

t will be noted that, within the range of fibres studied, as fibre diameter eases, the magnification of the axon changes from over 40% (fig. 8) to ler 20%.

Discussion

lthough much research has been based on teased preparations of peri-ral nerve, until recently the optical properties of the myelin lens system its apparent effect upon size relationships has not been appreciated. ple theoretical considerations and experimental models demonstrate that such effects undoubtedly occur. No quantitative assessment of these effec has hitherto been made.

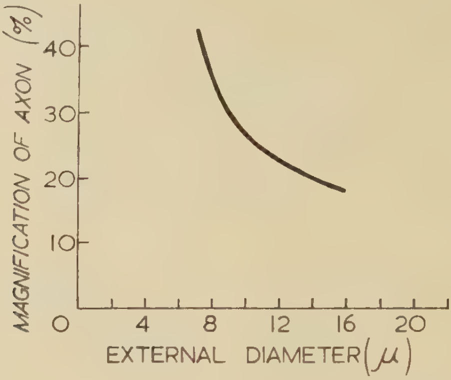
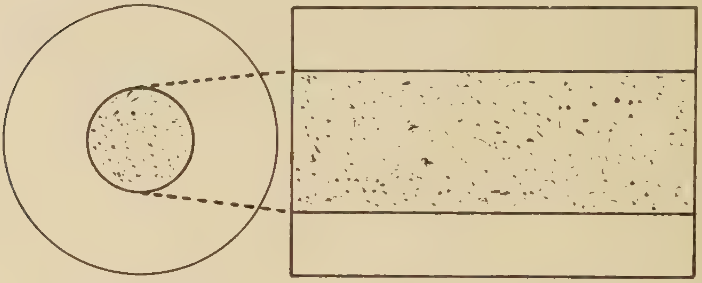


FIG. 7. Magnification factor. A curve relating % increase in apparent axon-diameter to external fibre diameter derived from fig. 6, B.



As seen in fresh-frozen transverse section

As seen in teased preparation

FIG. 8. Scale-diagram of a fibre, 7 μ in external diameter, from the NGM of the rabbit, illustrating the apparent change in dimensions when viewed in section and in a teased preparation.

It has been shown that the degree of magnification depends in part upo the ratio of axon diameter to sheath thickness. For a particular fibre size thi ratio differs in muscle and cutaneous nerve (Wendell-Smith and Williams 1958). Mixed nerves would clearly be unsuitable for such an investigation an in the present instance we have confined our studies to a muscle-nerve—th NGM of the rabbit.

In addition, in both teased preparations and sections our measurement have been limited to the uncomplicated internode. Variation introduced b the presence of shearing defects of Schmidt–Lantermann type and by th differences in thickness and curvature that may occur in the paranodal regio (Hess and Young 1952) has thus been avoided.

In the examination of teased fibres, possible effects of the immersio medium must be considered. These include a direct optical effect on th magnifying power of the lens system and an indirect effect due to changes i dimensions or refractive index of the myelin sheath. Finean and Millington 57) and Robertson (1958) have considered the effect produced by immer- n in fluids of different tonicity by X-ray diffraction techniques and electron roscopy. Both methods demonstrated that changes were slow to develop. e earliest changes were reported by Robertson, who noted splitting of the ipheral intraperiod lines after immersion in distilled water for 30 min. pertonic solutions (6–10 I Ringer) produced less change. After immersion 2 h the myelin was usually intact, distinct changes occurring only after re prolonged treatment (4 h). In the present investigations both teased es and sections were photographed immediately after immersion in a ticular fluid to minimize possible change.

Finally, variations in the ultra-structure and optical properties of myelin y exist (Schmitt and Bear, 1939).

n view of these multiple sources of variation, particularly the differences wn to exist between the nerves of muscle and skin, the magnification factor sented here for the NGM of the rabbit should not be applied without ervation to other sites and animals. However, the principles described may used to derive a magnification factor for a particular problem in hand.

n this connexion it has been demonstrated that the size relations of frozen tions prepared by the above technique do not differ significantly from se present in teased fibres. It is interesting to note that the sectioned fibres not exhibit the type of deformity observed by Lubinska (1956 *a*, *b*) in sed fibres of the frog. This was confirmed by observation of the tipped- r fibres (fig. 4, F, G, H). Furthermore, X-ray diffraction studies (Elkes l Finean, 1953) suggest that the ultrastructural relationships of peripheral ve myelin may be unaltered in the period immediately after freezing.

The present investigation has largely been concerned with preparations of elinated nerve-fibres after careful teasing in Ringer's solution. As we have icated, the use of such preparations in the fields of morphology and ysiology is widespread, and this makes a knowledge of their size relations atter of some importance.

To what degree do observations made on teased preparations reflect the tural state? The information on this subject is limited. De Renyi (1929), m examination of nerve-fibres in the nictitating membrane of the frog, ncluded that the operation of careful teasing is not necessarily accompanied size change. Nevertheless, a systematic study of this question is needed l is being undertaken in a number of vertebrate types by transilluminating hniques.

We wish to thank Professor R. Warwick and Dr. A. Nightingale for their erest in this work, Mr. A. N. Finch for technical assistance and photo- phy, and the Department of Medical Illustration, Guy's Hospital Medical hool, for preparing the illustrations. This investigation has been supported grants from the Central Research Fund, University of London, and from Endowments Fund, Guy's Hospital.

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The Central Nervous System in Larval Acraniates

By QUENTIN BONE

(*From the Marine Biological Laboratory, Plymouth*)

With one plate (fig. 5)

SUMMARY

This paper describes a part of the organization of the spinal cord of the larva of the crania, dealing chiefly with those tracts and cell groups that are probably concerned ith the control of the swimming pattern.

These observations serve as a basis for the comparison of the organization of the ord of the larva with that of the adult, and with that known for the larvae of the gnatha and higher vertebrates. Other observations are concerned with the arrange-ent of the peripheral nervous system, which differs in some respects from that f the adult. The first section deals with the arrangement of the fibre tracts in the cord, nd of the cell-bodies which give rise to these fibres.

The form and connexions of the giant cells are then described; it is shown that these ells are equivalent to the Rohon–Beard sensory cells of vertebrate embryos. They nd peripheral processes out through dorsal root nerves, but they differ from the ertebrate sensory neurones in their greatly enlarged dendrite field and the longitu-inal axon in the cord.

Thirdly, the innervation of the gill musculature is described. A ventro-lateral symmetrical nerve is found, formed by the junction of fibres from the right anterior orsal root nerves.

Lastly, the arrangement of the whole system is discussed in relation to the systems und in the larval stages of other primitive chordates.

INTRODUCTION

MUCH is known of the large neurones concerned with the control of swimming patterns in the early stages of several groups of vertebrates; e studies begun by Coghill (1914) upon urodele embryos initiated work upon mprey and elasmobranch developmental stages, from which theoretical nclusions have been drawn regarding the presumed primitive condition of e chordate central nervous system. The argument is briefly summarized y Whiting (1957) in a discussion of the Mauthnerian system of neurones the pro-ammocoete larva of the Agnatha.

Nothing has been reported of the structure of the nervous system of the rva of the Acrania, in many ways the closest living animal to the primitive ordate; it is this larva which would perhaps be expected to provide most formation about the structure of the spinal cord of the ancestral vertebrates. he structure of the cord in adult Amphioxus is fairly well known (what is own is summarized in fig. 1), but there still exists confusion in the litera-re regarding the homologies of the dorsal root ganglion cells, and even out the existence of spinal ganglia (see Lele, Palmer, and Weddell, 1958, 433); the functional divisions of the longitudinal tracts in the cord are not

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yet clear. In the larva, conditions are less complex than in the adult, an although I have not had the opportunity to examine living larvae at the stag described in this account, something can be inferred of the functions of th system.

MATERIALS AND METHODS

The majority of the observations recorded in this paper were made upo larvae of *Branchiostoma belcheri* Gray, which Mr. J. Wickstead collecte from the Singapore plankton. An account of the ecology of the larvae an details of their collection will be published elsewhere (Wickstead and Bon 1959). I am much indebted to Mr. Wickstead for allowing me to examine thes specimens. The largest larvae were 5·3 mm long, just postmetamorphi the smallest 3·6 mm long, with 10 gill slits. In this species, metamorphos takes place when 19 to 21 primary gill slits have been achieved (as compare with 14 to 15 in *lanceolatum*); the process will be described in a subsequen communication. In addition, larvae of the species listed below have bee examined after reduced silver staining:

Paramphioxus bassanus (Gunther)
Amphioxides pelagicus (Gunther)
Branchiostoma lanceolatum (Pallas)
B. malayana Webb
B. nigeriense Webb
B. takoradii Webb

I am most grateful to Dr. A. S. Fuller for material of *takoradii* and *nigeriense* and to Prof. J. E. Webb for material of *nigeriense*.

These other species did not yield preparations as informative as those from *B. belcheri*, but they sufficed to show that there was no essential differenc in any of the species from the arrangements found in *belcheri*; it is mos probable, therefore, that the arrangements described in this paper ar characteristic of the Acrania as a whole.

The larvae were stained as whole mounts, by a variant of Holmes's silve method (Holmes, 1947), and then examined as whole mounts or sectioned Successful results were obtained from larvae fixed in sea-water formaldehyde in sea-water Bouin, and in mercuric-formaldehyde; no useful results wer obtained from larvae fixed in 70% ethanol or in Kleinenberg's fluid. Fixatio was not the only variable, however, for other larvae fixed in sea-water formaldehyde gave no results; and in a batch of larvae fixed and treated in the sam way, considerable variation in staining was found. Such variation prove useful in the examination of whole mounts, for in different specimens differen parts of the system were stained, and a reasonably complete picture could b built up by comparison of various larvae.

Most of the observations in this paper have been made upon the whol mounts. Twenty-three larvae were mounted after successful staining; 6 serie of larvae were also sectioned.

RESULTS

The arrangement of some of the cell groups in the adult Amphioxus cord seen in fig. 1. Only a few members of each cell type are shown in the iagram, but the numbers of dorsal bipolar cells (A cells) and viscero-motor ells are approximately correct. The diagram is constructed from the observaons and figures of Rohde (1888), Retzius (1891), Johnston (1905), Stendell 914), and Franz (1923), and from original observations upon whole mounts nd sections. The arrangements are, briefly, as follows. The sensory systems of cells are best known. Of these, the dorsal bipolar cells of Retzius (A) form a more or less continuous column along each side of the central canal near the roof of the cord. Their axons pass along the cord, and pass out by T- and Y-branches in the dorsal root nerves. These cells are much more abundant in silver preparations than in Retzius's figures from methylene blue preparations. Almost certainly, it is processes from these cells which form the greater part of the nerve-fibres terminating in the skin.

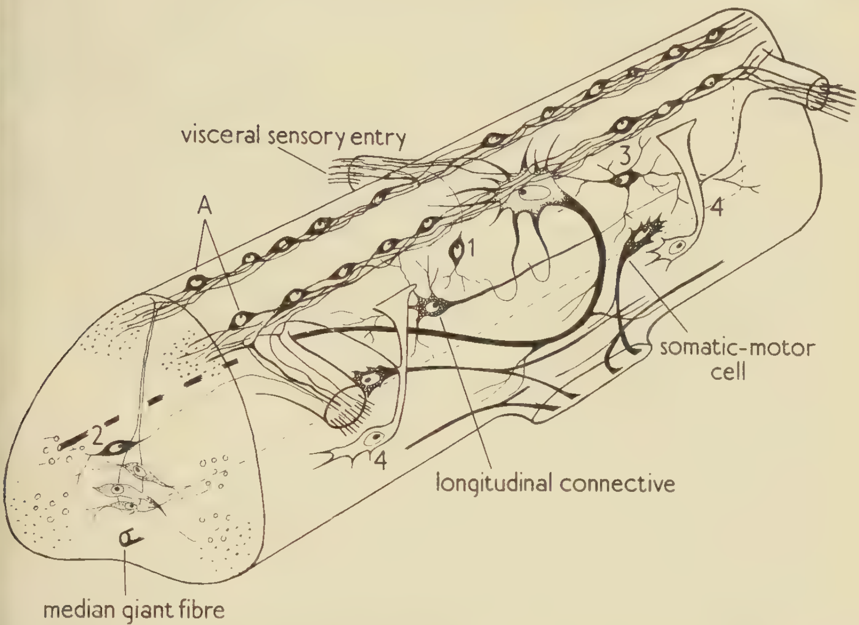


FIG. 1. Stereogram illustrating the structure of the spinal cord in adult Amphioxus. Apart from the A cells of Retzius and the type 4 cells of Johnston, the relative abundance of the cell types is not indicated in the diagram. For explanation of lettering, see text.

Secondly, there are other types of cell sending fibres out in the dorsal root nerves, which are distinguished by the position of the cell-body and dendrites relative to the central canal; these Johnston divided into his types 1 and 2; Retzius figured another type shown at 3. Johnston regarded these cells types (together with Retzius's A cells) as equivalent to the dorsal root ganglion cells of vertebrates. He added to these another cell type also equivalent to the vertebrate dorsal root ganglion cell, and more similar to it in that the cel body lay actually in the dorsal root or even a short way along the course the dorsal root nerve. I have omitted this type (Retzius never saw it) as am not convinced of its existence (see also Lele, Palmer, and Weddell, 1958

The last of the sensory systems known for the adult cord is formed by th giant Rohde cells. These have a very characteristic axon arrangement (mo accurately described by Rohde); they have also a characteristic dendri system like that of the larva. It is most probable that some at least of the

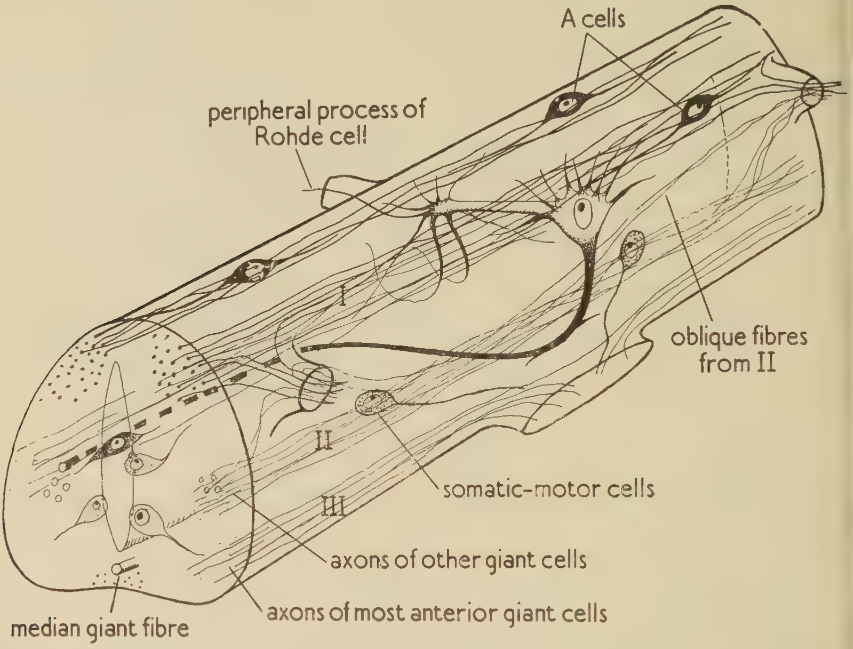
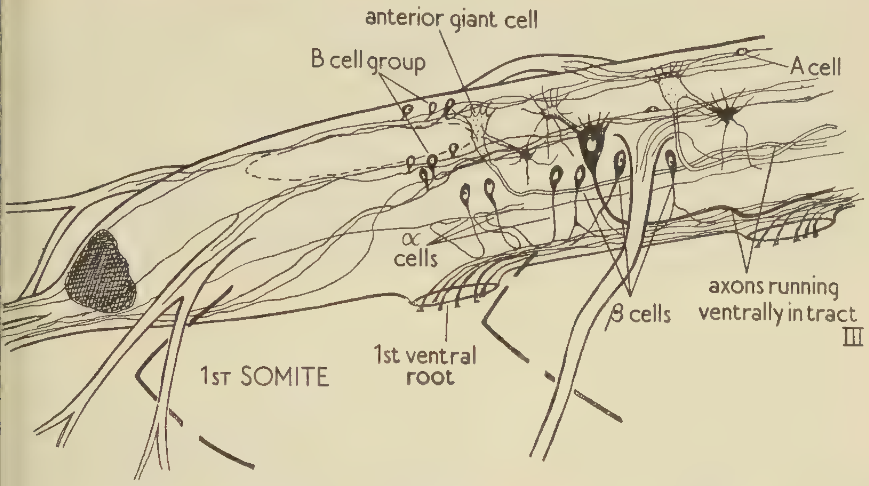

A cells
peripheral process of Rohde cell
I
oblique fibres from II
II
somatic-motor cells
III
axons of other giant cells
axons of most anterior giant cells
median giant fibre

Fig. 2. Stereogram illustrating a section of cord in the larva, comparable to that seen for t adult in fig. 1. Note numbers of A cells, the extent of the dendrite field of the Rohde ce and the oblique course of fibres of tract II rising to the roots. The somatic motor cells ha not been definitely identified.

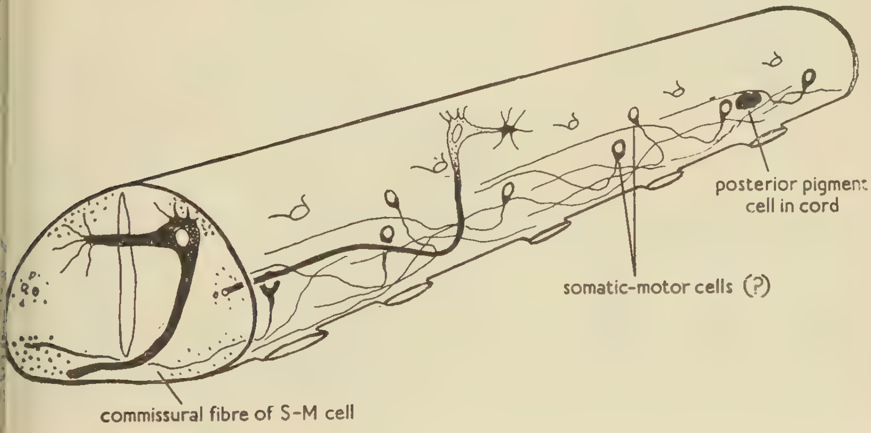
cells possess a peripheral process in the adult, which passes out through dorsal root.

Much less is known about motor, internuncial, or associative systems in th Amphioxus cord. Visceral motor cells were recognized by Johnston (type 4 These sent a large wide process to the cord roof, (where it ended in a fa shaped expansion); from this process an axon passed out in the dorsal root Such cells as seen in reduced silver preparations are arranged in a more less segmental manner, one per segment.

Somatic motor cells have been described by Stendell (1914). They lie at different level in the cord from the ventral roots (so that cell-bodies are n seen in the same section as the ventral root), and send their axons along th cord for a short distance; then they pass out by the ventral root branchi



G. 3. Stereogram of the anterior region of the central nervous system in the larva. Note B- l group; termination of tract III at first ventral root; cells at α giving rise to com- ssural fibres; cells at β contributing to tract III; and most anterior giant cells and their axons.



G. 4. The caudal end of the larval cord. Note the possible somatic motor cells and their crossing collaterals (?), and the irregular course of the fibres in tract III.

give off a collateral before they pass out, much as Coghill described for *mblystoma*.

Finally, there are many cells in the cord which cross the central canal, at l levels; some are shown in the diagram. Probably some of these fall into e groups so far considered, but others are most likely to be internuncials various types. It is evident that there must be many other functional units the spinal cord of the adult about which we know little: not much is known the different swimming mechanisms which Ten Cate's (1938) work revealed;

nothing is known of the way in which the extraordinarily rich sensory outg from the atrium is dealt with in the cord (Holmes, 1953; Bone, 1958).

Fig. 2 illustrates a comparable portion of the cord of the larva; figs. 3 an show the arrangements at head and tail in the larval cord. At this stage it sufficient to point out that much of the adult organization has already be achieved by the larva before metamorphosis, but there are certain nota differences, particularly relating to the A cells of Retzius, the anterior B ce described in this account, and the apparent absence of visceral motor ce similar to those in the adult.

The fibre tracts in the larval cord and the location of the cell-bodies giving r to them

In the mid-region of the cord there are 3 distinct longitudinal fibre trac lying laterally to the nerve-cell bodies adjacent to the central canal. They a seen in fig. 5, D–F. In addition to these 3 tracts, there are less defined bund which run in the lateral part of the cord, and in the mid-ventral line bel the central canal.

The latter bundles grade to a certain extent with the two most ventral the fibre tracts; there is not a very distinct division between the lower tra (II and III) and these other fibres. The dorsal tract (I) is probably coars than other fibres in the cord; this tract can be seen easily in whole moun (as in fig. 5, A).

The fibres of tract I contribute to the dorsal root nerves, and can be trac out under the skin, some passing down so far as the metapleural fol Johnston recognized these coarser fibres (they are very similar in arrang ment in larva and adult), and supposed them to be general cutaneous fibr pointing out that the way they ramified after entering the cord (as seen his Golgi preparations) suggested that they might play some part in t

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FIG. 5 (plate). (The 100-μ scale refers to A, B, D, E, F, 200-μ to C and G.)

A, anterior end of cord, whole mount. The dorsal B cells are just visible. An axon of o such cell makes a noticeable curve anteriorly; the axon of a β cell passes downwards p this curve. Posteriorly the axons of the 2 of the most anterior giant cells are visible; t labelled 1 is the fourth giant cell of the cord.

B, a B cell from another specimen. Whole mount.

C, section of mid-region of cord to illustrate double origin of dorsal root nerves from tra I and II.

D, section at an anterior level showing paired ventro-lateral cells, possibly somatic visceral motor. Dorsally, commissural cells connect the sensory columns of both sides. dorsal commissural cells; *s*, possible somatic or viscero-motor cells.

E, section just posterior to D, showing ventral commissural fibres. In this and the previ section, tract I appears double because it has at this level divided into the 2 rami that will g rise to nerves 1 and 2.

F, section from more posterior level showing the ventral ramifications of the dendrites the Rohde cell amongst fibres of tracts II and III.

G, whole mount showing oblique ascent of giant fibre of cell on the left side of the cord, p the beginnings of the axon of the next cell on the right. The pigment cells are out of foc as are the cell bodies of the two Rohde cells. *g*, beginning of giant cell axon; *cl*, oblic ascent of fibre from contra-lateral giant cell.

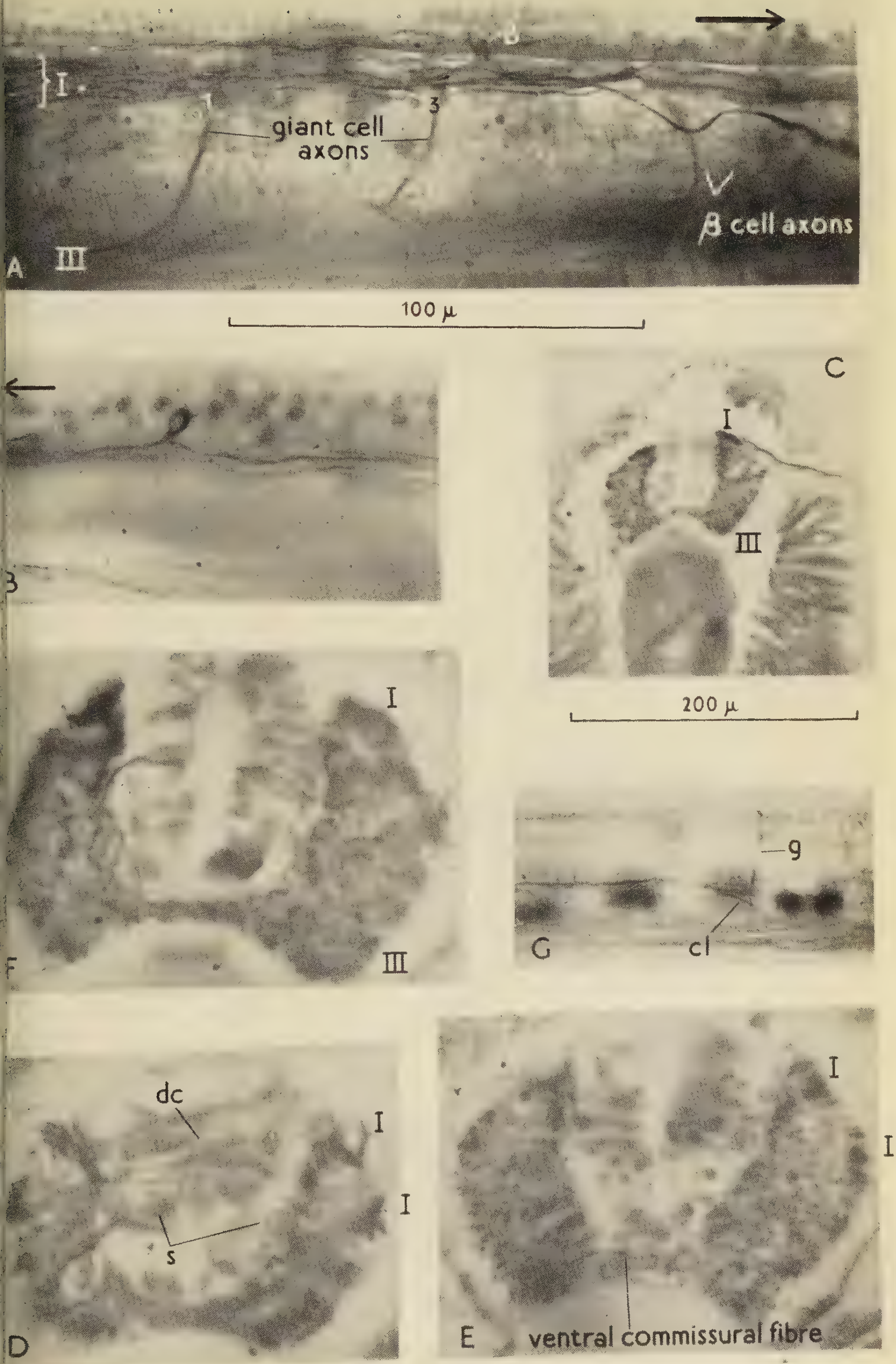

I
β
giant cell axons
3
β cell axons
A
III
100 μ
C
I
III
200 μ
B
I
F
III
g
G
cl
dc
I
I
s
D
I
I
E
ventral commissural fibre

FIG. 5

Q. BONE

ative independence of the segments in locomotion. In transverse section,
cell-bodies giving rise to these fibres are never seen in the same plane as
dorsal roots themselves. In whole mounts the fibres in tract I may
netimes be traced for long distances (up to three-quarters of the length
the cord). Two different cell types contribute to this dorsal tract. Other
es may do so, but if this is the case, their connexion with tract I has not
en observed. The first of these types, the A cells, are found at intervals all
ng the length of the cord. They are bipolar, sending processes in each
ection which can only be followed for a segment or two in tract I; most
bably one process soon passes out of an adjacent dorsal root, the other
apsing with dorsally placed internuncial or motor-cell dendrites. It is
dent that these cells are equivalent to the A cells of the adult, described
Retzius. But they differ from the adult condition in that they are much
s numerous, and more irregular in arrangement; evidently at metamor-
osis or just before it, the innervation of the skin is not so developed as it
er becomes in the adult. Towards the tail, near the extreme posterior end
the cord beyond the level of the anus, there are cells similar in position to
A cells, but they possess only one process, which is directed caudally
g. 4). Presumably it passes out of the cord by the most posterior dorsal
ots. At this level, many of the cells in the cord have scarcely differentiated
d have the appearance of neuroblasts, though fibres from more anterior
lls passing to the cord tip are well stained; processes from cells in this
gion are rarely seen. It is possible that these unipolar cells may represent
stage in the formation of the A cells rather than another cell type, and that
ey later grow out another long process to conform with the more advanced
cells of the adult and of the more anterior regions of the larva.

The second cell type contributing to tract I is found at the anterior end
the cord only. These B cells lie in a special dorsal group at the extreme
ont end of the cord, just posterior to the cerebral vesicle. They are seen in
. 5, A, B. Like the A cells, which they resemble, the B cells are 'bipolar',
it the cell-body lies to one side of the axons, so that these cells much
semble vertebrate dorsal root ganglion cells. One process passes out through
e rostral nerves and can be followed out to the edge of the rostrum. The
her runs caudally in tract I, and in some cases may be followed beyond the
vel of the anus.

Like vertebrate dorsal root ganglion cells, a series can be found showing
e way in which these cells arise from normal bipolar cells like the A cells;
the oldest larvae some of the cells are unipolar, and the process bifurcates
some distance from the cell-body.

Not only are these cells similar in development and form to the vertebrate
rsal root ganglion cells, but they may be equivalent also in their peripheral
nnexions, being connected with a peripheral sensory neurone. The peri-
eral process of the B cell passes out along the rostral nerves to the edges
the rostrum where are arrayed the mysterious corpuscles of de Quatrefages
847), and appears to be in connexion with these corpuscles.

These sensory corpuscles have long been known, but very little is kno of their structure or function. Probably they are sensory (de Quatrefa thought they secreted mucus), and respond to touch, but this has not b shown experimentally. They appear to have been regarded previously primary sense cells of the invertebrate type, sending their axons to the c through the rostral nerves (e.g. Franz, (1927): 'Die Quatrefagessch Körperchen der Rostralflosse sind kleine periphere Ganglien'). Other su peripheral neurones are known elsewhere in Amphioxus, especially in atrial system (Holmes, 1953; Bone, 1958*a*). However, consideration of arrangement of these cells makes it possible that there is a peripheral syna between the axons of the B cells and the sensory corpuscles. Most of fibres stained in the rostral nerves are traceable to B cells in the dorsal gro These fibres can be followed to the edge of the rostrum where the corpusc lie. The relative numbers of the axons in the rostral nerves, the numbers B cells, and the numbers of sensory corpuscles accord with the hypothe that there is a peripheral synapse.

Unfortunately in neither larva nor adult have I been able to make out t nature of the connexion between the corpuscle and the rostral nerve axon but the suggestion that they are not primary sense cells gains support fr consideration of the function of the B cells.

It is clear that these cells in the larval cord represent a system adapted convey sensory data obtained from the rostrum to the whole length of t spinal cord. In this respect the B cells are analogous, but not homologo to the special Mauthnerian co-ordinating systems of lower craniates.

It is probable that the long caudal processes of these cells are in connexi with the segmentally arranged systems of cells directly controlling the swi ming pattern in each segment. If this view be correct, then the B cells a organized so that stimuli received at the front end of the animal can evo rapid, co-ordinated swimming movements.

The Mauthnerian systems of the Ichthyopsida relate the informati received by the acustico-lateralis system to the swimming movements; Amphioxus there are no special sense organs or lateralis system; it seems th touch (?) stimuli from the anterior region are important to the animal a that these evoke swimming movements through the B cell system. Lit is known about the habits of acraniate larvae, but in *B. belcheri* it seen probable that the larva feeds on the bottom during the day and rises to t surface at night, perhaps to escape predators (Wickstead and Bone). T larva may remain in the surface layers by the action of its body cilia (Wille 1891), and probably is inactive when on the bottom, so that except duri the journey to the surface, muscular locomotion is unlikely; touch stimuli the rostrum thus would evoke activity on the part of a normally quiesce larva.

Both A and B cells (the B cells possibly to be regarded as a specializ derivative of the A cells in the anterior region of the cord) and their process form the somatic sensory division of the larval cord. In arrangement, apa n the absence of reports of the B cells in the adult, the system is similar oth larva and adult.

n the adult, in addition to Retzius's A cells, Johnston described others ch he regarded as unquestionably homologous with spinal ganglion cells of ebrates. One of these types we have rejected, the cells Johnston figures ne roots themselves; no other author using specific techniques for nervous ne has ever found these cells, and it is most likely that in his Golgi pre-ations some confusion has arisen with sheath cells. However, Johnston's ervations are elsewhere so reliable that it may yet be shown that he was ect in this instance also.

he other types of cell have not been identified in the larval cord, but this is to say that they have not differentiated or that they are not present; I have been able to observe them in my preparations. The question of homologies ween vertebrate spinal ganglion cells, and any of the cell types in Amphioxus ch have been observed to send fibres out through the dorsal roots is not mple one; for it is necessary to consider what stages are to be regarded omparable in the two groups. Some of the problems involved will be dis-sed in a later section of this paper.

he other tracts, II and III, which are recognizable in sections of the cord, less easily referable to specific cell groups and functions.

ract II, lying just below the dorsal tract I described above, is made up of r fibres, and contributes such fibres to the dorsal root nerves. These fibres e the cord more laterally than the main root of the nerve, and are less nerous; fig. 5, C shows the way in which these fibres reach the dorsal root. is division in the larva of the dorsal root nerves was long ago noted by ymans and van der Stricht (1898) (who stated that the dorsal strand was ular, the dorso-lateral fibrous in origin); they did not analyse the composi-n of the two components further. The smaller size of these fibres and their tive sparseness in the dorsal roots suggests that they may possibly be ıer visceral sensory or visceral motor components.

n the adult Amphioxus, visceral sensory fibres pass in through the dorsal ts, and are derived from the system of neurones lying in the atrium under atrial epithelium. These neurones are not found before the formation of atrium, and appear to migrate out along the dorsal root nerves in the course netamorphosis (Bone, 1958*a*).

The adult visceral sensory system has therefore not yet developed in these vae. The fibres of tract II are probably best regarded as visceral motor. ere is a large visceral motor component in the adult which supplies the rygial muscle flooring the atrium (Heymans and van der Stricht gave a d account of the system); these axons pass out through the dorsal root ves, from cells in the cord. Johnston regarded his cell type 4 as visceral tor (see fig. 1) on general grounds of similarity of cell position to the cells he visceral motor column in fishes; in my own silver preparations of whole unts of the adult cord, these cells are not so numerous as would be sup-ed were they to form the only visceral motor component contributing to the pterygial innervation. As Dr. H. P. Whiting has pointed out to me, ii probable that Johnston's type 4 cells form only a part of the visceral mc 'centre' in each segment of the cord, around which other, smaller, visce motor cells are arranged; these other cells have not yet been recognized.

In the larva there is no pterygial muscle (at metamorphosis it forms as atrium floors in from behind forwards), but there are various unstria visceral muscles which control the shape of the prae-oral pit, the mo and front of the pharynx, and the degree of contraction of the gill apertur

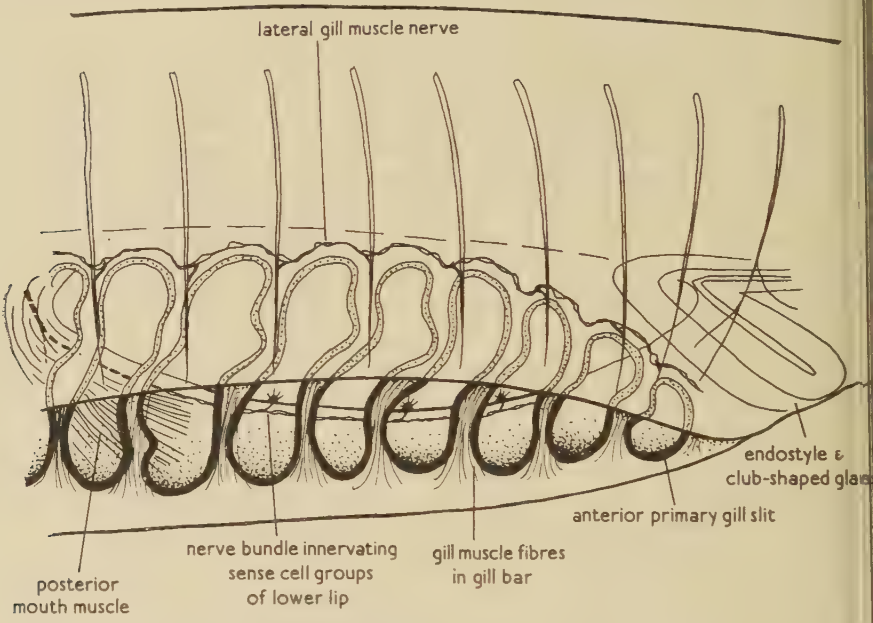

lateral gill muscle nerve
endostyle & club-shaped gla
anterior primary gill slit
posterior mouth muscle
nerve bundle innervating sense cell groups of lower lip
gill muscle fibres in gill bar

FIG. 6. Diagram of the asymmetrical innervation of the gill muscles in the larva.

All these muscles are adaptations developed in relation to larval life; th disappear at metamorphosis, and their main function of controlling the r of flow of water through the feeding apparatus is taken over by the striat muscles of the atriopore and velar sphincters. The smooth muscle-fibres the larva are innervated by fibres passing out through dorsal roots. Th manner in which these fibres are arranged is of interest.

At the anterior end of the pharynx, and possibly throughout the gill seri the muscles of the gill openings are innervated by axons passing out throu dorsal roots of the *right* side only. These axons run down the large rig metapleural fold, and bend inwards to form a lateral nerve looping over t gill openings in the way shown in fig. 6. At the anterior end of the system fibres from the *left* dorsal root nerves pass to the gill muscles. Posterior to t 8th or 9th gill slit it is possible that *left*-side dorsal root nerves contribu axons to the ventro-lateral gill nerve, but I am inclined to think this unlike and to suppose that the whole series of gill muscles is innervated solely fro *right* dorsal roots. When the visceral ramus of the dorsal root nerve of the *ht* side reaches the ventro-lateral gill nerve, axons pass both anteriorly and steriorly in the lateral nerve, so that it appears probable that the system s as a whole, all gill slits contracting, or all relaxing, rather than that the va is capable of contracting separate slits individually.

The anterior dorsal root nerves of the *left* side probably also contain ceral motor-fibres. Anteriorly, on the left side, there are muscles surnding the prae-oral organ, which change the shape of its opening; there muscle-fibres which alter the shape of the mouth opening anteriorly and steriorly; and there are muscle-fibres surrounding the pharyngeal wall ntrally which can alter its shape. Legros (1910) spoke of these muscles as éments musculaires striés', but they are unstriated like the gill muscle-res. It is probable that dorsal roots of the left side innervate these muscles, t, with the exception of the posterior mouth-muscle, I have not been able see this innervation in my preparations.

The left anterior dorsal root nerves also contain sensory fibres in connexion th special groups of sensory cells on upper and lower lips.

At intervals along the lips are found groups of cells bearing extremely long ia; nerve-bundles terminate in relation to these cells, and it is almost rtain that they are either chemoreceptors, or sensitive to touch (responding particles of different sizes entering the mouth). It is probably these cells ich are responsible for the ability of the larva to respond to unsuitable terial entering the mouth by closing the gill slits (Bone, 1958*b*). The cell ups of the lower lip are innervated by the dorsal root nerve which passes wn just posterior to the hinder end of the mouth, and sends a bundle of res forward along the edge of the lower lip.

The asymmetry of the anterior visceral motor innervation in the larva ses theoretical questions of interest. The gill openings that are supplied by lateral nerve formed by axons from *right* dorsal root nerves, are those of primary series. That is to say, they are those gill slits which upon metarphosis will migrate round to the *left* side of the pharynx, and form the finitive *left* series of slits. The adult right side slits have not yet appeared any of the larvae which have been selectively stained for nerves; they are rived from secondary slits formed high up on the right side of the pharynx.

In other words, in the acraniate larva morphologically *left*-side structures innervated by *right* dorsal root nerves.

In the adult, it has been shown that part of the buccal region is innervated an asymmetrical way, *left*-side nerves passing to the right side of the oral od (van Wijhe, 1893). In adult structure, asymmetry is related to the ginal asymmetry of the mouth of the larva (lying on the left side), and the nner in which symmetry is secondarily achieved at metamorphosis. It ght be supposed that in the larva asymmetry of innervation of the gill ies would be related to adult symmetry, i.e. that the gill series would be ervated by *left*-side nerves; this is not the case. It seems best to regard the mmetry of gill innervation in the acraniate larva as a larval character only, bearing no relation to adult structure (the muscles innervated disappea metamorphosis); perhaps it is dictated by the asymmetry of the mo which would make it difficult for left-side nerves to reach the more ante gill muscles.

The larval gill- and mouth-muscles are unstriated, innervated by fil passing out through anterior dorsal roots. The arrangement is not un that of the parasympathetic outflow of the craniate, where autonomic fil pass out through the (morphologically) dorsal anterior roots of VII, IX, X. But it is preferable not to attempt any comparison here, for several reas and I do not feel that the dorsal root efferents either in larva or adult can equated in a simple way with any part of the autonomic system of the crani

I have not been able to identify with certainty the cell-bodies whose ax make up tract II. There are many cell-bodies lying laterally to the cen canal, at the level of this tract, and which send processes to it (as in fig. 5, but I have not been able to trace these processes in whole mounts.

Tract III, which lies ventro-laterally and is made up of medium-si fibres, is the somatic motor column. Its fibres leave by the ventral ro none pass forwards past the most anterior ventral root of somite I (fig. so that it is clear that there are somatic motor-fibres only in this tract. In anterior part of the cord there are crossing fibres which link the two som motor columns of each side of the cord; some of these chiasmata are s in fig. 5, E.

Farther along the cord, such chiasmata are not observable until the p anal region near the tail; however, along most of the length of the cord, most ventrally placed cells send processes to the columns of each side of cord.

As with tract II, again with the somatic motor column, it is a diffic matter to identify the cells of origin of the tract.

Most anteriorly a few cells have been found in several different specim which contribute fibres to the contralateral tract III. These are illustrated α in fig. 3. Others in the same region (β) are in relation to tract III, sendin process down to it from a rather dorsally placed cell-body, the process f quently being observed to bifurcate about the level of tract III. Rather simi cells can be occasionally observed along most of the first three-quarters of cord.

In the anterior part of the cord at any rate, the somatic motor cells cann be so clearly differentiated as they are in the pro-ammocoete larva (Whiti 1954, 1957), where they are large and easily recognizable at an early sta Stendell's account of the somatic-motor cells in the adult (see fig. 1) show that there they were large, and lay at a different level in the cord from t ventral roots, by which their axons issued. This makes such cells diffic to recognize in sections, but it is possible that such cells as those seen fig. 5, H are somatic motor.

In the posterior region of the cord conditions are different, in some spe mens at least, for there, segmentally arranged large ventral cells are foun ich may possibly be somatic motor. They lie at the level of each ventral t, i.e. in between the dorsal roots (fig. 4), and send a rather thick process trally which curves across the floor of the cord and passes anteriorly. No cesses from these cells can be followed out through the ventral roots ntral root fibres have not stained in any specimens examined). It is possible t these cells may send off an axon at the level of the root, and that the thick cess passing forward may be a collateral, but this I have not been able observe in my preparations. It is interesting that the collateral (if such it can be followed forward for some distance in the cord (several somites) d that it crosses the floor of the cord. In position these cells are similar to somatic motor cells of other early craniate larvae, and the formation of n collaterals would be characteristic not only of the adult, but also of her larvae (e.g. *Amblystoma*, Coghill (1914)).

That such cells should be found only in the tail end of the cord may be plained on the basis that the tail of the larva is less advanced than the head: larva is 'younger' at tail than head. In several quite different features g. the development of gill slits, nephridia, and spinal cord), the more sterior regions of the body are relatively retarded with respect to the more terior. In the spinal cord, embryonic features are seen in the most caudal rt of the cord when the more anterior cells have lost the appearance of uroblasts.

If the acraniate larva is like the pro-ammocoete larva of the Agnatha, then is possible that it does possess large, easily recognizable somatic motor urones, but that except at the most posterior part of the cord, these cells ve already been altered or replaced by the development of a more diffuse stem of smaller cells when the larvae have reached the stage examined in is study. This conclusion may be supported by consideration of the activity the acraniate larva; that it begins muscular movement at the neurula stage d is capable of organized swimming before the single-slit stage (several eeks at least before the stage described in this account), suggests that the gmental neurone pattern concerned with swimming movements may have en partially replaced by the time that metamorphosis approaches. However, e absence of easily recognizable large somatic motor cells in the larva amined is certainly a striking difference from the accounts given for craniate rvae, whatever the explanation for it may be.

ie Rohde cells

In the adult, Rohde gave an excellent account of the giant dorsal cells at bear his name. He figured a horizontal section of the cord, illustrating the y in which the axons of the cells passed up and down the cord, and he o showed that these cells were absent from segments 12 to 39. This last servation was taken advantage of by Ten Cate to show that the giant ls were involved in one of the three swimming patterns which he dis- guished. In the larva, the arrangement of the Rohde cells is slightly different m that in the adult, both in their form and their distribution in the cord.

In the adult, the most anterior giant cell sends its axon down the middle the cord, below the central canal, to form the median giant fibre (figs. 1, This cell is larger than any of the others, and its axon larger in diameter; axon does not cross the central canal in the manner of the axons of the ot giant cells (figs. 1, 2).

A similar cell is found in the larva, but there are 5 giant cells anterior to which differ from those found elsewhere in the cord in that their axons not cross below the central canal, but run backward in the cord on the sa side as the cell-body. These relations are seen in fig. 7; some of the mo anterior cells are seen in fig. 5, A.

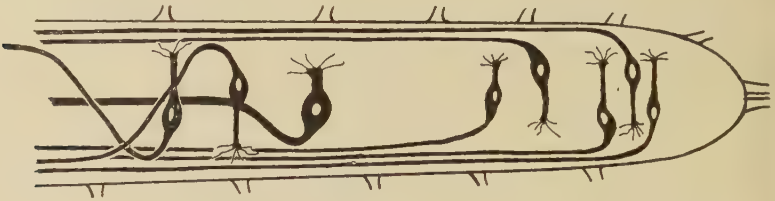


FIG. 7. Distribution of giant cells at the anterior end of the cord.

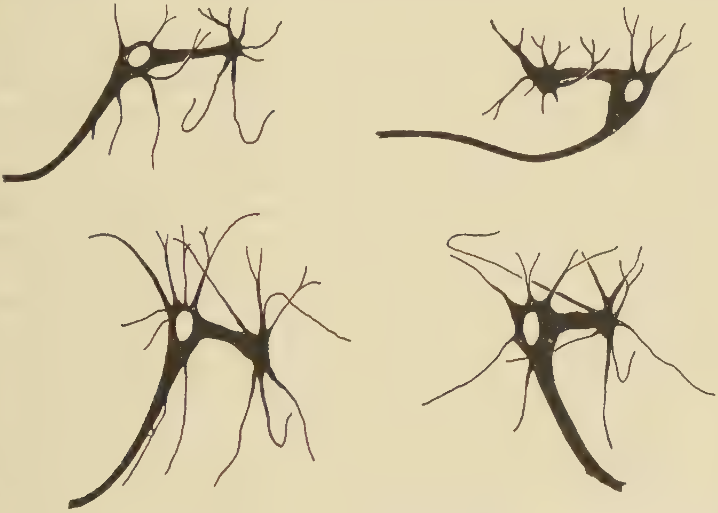
Rohde stated that he had observed large cells anterior to the first giant c that he described, but he could not observe their processes. If there is difference between larva and adult (i.e. if Rohde's more anterior cells do n correspond to these cells of the larva), then it is possible that it is due to specific difference, or else these more anterior cells may regress duri adolescence. It is evident that their connexions must be unlike those of t other giant cells, but I have not been able to make out anything of this in m preparations. In every respect except the arrangement of their axons th appear identical to more caudal Rhode cells.

As in the adult, the more anterior cells send their axons caudalwards, t more posterior cephalad; there is also a gap in the mid-cord region where th are not found. Even in the most posterior regions of the cord the giant ce are well developed, with a full array of dendrites, whereas other cells in th same region have scarcely begun to differentiate and still appear as neuroblas only.

Evidently, the system appears early in ontogeny, and persists into adu life; this view is in harmony with the extremely precocious development swimming movements by the larva and with Ten Cate's results demonstrating the significance of the system of giant cells in normal swimming.

The organization of typical examples of these cells in the larva is seen figs. 8; 5, F; and 2.

The cell-body lies dorso-laterally in the cord, usually near a dorsal roo and gives off a large axon which curves under the central canal and rise obliquely to pass longitudinally at the level of the top of tract III (fig. 5, G Fine collaterals are sometimes seen from this axon, but in distinction t those observed by Retzius, these seem generally to be in connexion wit e fibres of tract II, than with the somatic motor column, that is, they were in nexion with visceral motor fibres. No other connexions have been erved, but it is certain that there must be connexions with tract III; as the adult, these are probably axo-axonal. The cell-body gives off dorsally naracteristic fan of dendrites, which rise into tract I, and evidently connect h its fibres over a wide area in the segment. Possibly some of these proses may pass out through an adjacent dorsal root, but I have not observed



. 8. Diagrams of the arrangement of Rohde cell processes at various levels in the cord.

m to do so. Laterally, the giant cell sends a thick process to cross the tral canal, which expands on the opposite side to form a mass almost as ge as the cell-body, from which numerous processes are given off. These cesses radiate dorsally and ventrally (the ventral ramifications are seen in t in fig. 5, F), and connect with all the fibre tracts in the cord. There are en a pair of larger ventrally directed processes which pass inside tract II d then curve upwards to rise up more laterally to it. This is probably aracteristic of the Rohde cells of larva and adult. The significance of this angement is not known.

Most significant of all, one or more of the processes on the contra-lateral e of the canal to the cell-body pass out through the dorsal root opposite cell-body. This observation of the peripheral process of the Rohde cell the larva confirms the earlier statement by Heymans and van der Stricht 98) that 'chez des larves de $5\frac{1}{2}$ mm. nous avons observés plusieurs cellules sales [the Rohde cells] dont un prolongement pénètre directement s la racine dorsale voisine'. As they considered the Rohde cell to be tor (because of its resemblance to an anterior horn cell in the craniate d), the significance of their observation was not understood. As far as I am are, no later worker upon these cells has considered their work.

Unfortunately I have not been able to follow the course of these periph processes, but it is probable that they pass to the skin; possibly in the ante region of the body to the metapleural folds.

The giant cells of the larva are thus in connexion with all the tracts in cord, and also send out a peripheral process. This gives them something common with the Rohon–Beard sensory neurones, familiar in the ea ontogeny of craniates; consideration of their title to this rank will be defer to the next section of this account.

DISCUSSION

The observations recorded in this paper are incomplete, and they re only to a single, late stage in the life of the acraniate larva. It is unfortun that material of earlier larvae has not been studied, for interpretation of so of the arrangements in the cord of these late larvae must remain uncert until earlier stages are examined. There are, however, certain points t seem clear. First, it may be suggested that the Rohde cells are the functio equivalent of the Rohon–Beard cells of the larvae of the Ichthyopsida. Th are differences from the classical descriptions of these cells (discussed Hughes, 1957), but these seem less striking than the similarities. In the la at least, the Rohde cells give off a peripheral process that runs out throu a dorsal root passing probably to the skin. It is possible that a branch m pass to the muscle-blocks, but this has not been observed. The cells are ve large, lie dorsally in the cord, and send processes in relation with soma sensory columns. But these similarities, and the fact that, like the *Hinterzel* of the adult lamprey, these large cells persist until the adult (perhaps losi their peripheral process), exhaust the points of resemblance of the Roh cells to Rohon–Beard cells. They differ from Rohon–Beard cells in the exte of their dendrite fields (in all columns of the cord), in their direct relatio with somatic motor cells (through axon collaterals of both cell types) witho the intervention of internuncials, and in the longitudinal extent of their ma process in the cord.

It is here that information about earlier stages is desired, for only fro earlier stages could we be certain that we are dealing with true Rohon–Bea cells as they appear in all the lower craniates. It was suggested above th the development of these cells and all their processes in the most cau region of the cord, where other cells are as yet only neuroblasts, implies th they form a system that appears early in ontogeny, and that thus they mig be regarded as more or less in their primitive condition even in the larv that were studied. But if this is so, then there are differences from the Roho Beard cells of the classical craniate type, even at an early stage. Perhaps wi our present lack of knowledge of the earlier stages, it is best to regard t Rohde cells in Amphioxus as a system that appears early but persists the adult and which is in some ways functionally and structurally similar to t Rohon–Beard system of transient ganglion cells in the craniates, but is n homologous with the latter system.

It is tempting to speculate upon many interesting questions that the ganization of these cell systems raises, e.g. whether one is to be regarded as rived from the other, or whether the absence of internuncials, and the ect connexion between sensory and motor cell is primitive, or is an acraniate ecialization. But such speculations cannot be profitable until much more known about the earlier larva of the Acrania. It is more or less clear that e patterns of nerve-cells found in the early stages of the lower craniates e homologous, and that they represent ancestral stages (perhaps more or s modified) in the development of the central nervous system of the aniates. It is not surprising that the ancestral conditions should be recapitu- ted in modern ontogeny, for it is no doubt both economical and convenient r the modern larva to build its patterns of nerve-cells upon the ancestral tterns in each segment.

But the picture that this view gives of the ancestral condition of the spinal rd does not accord with that found in the acraniate larva.

In the acraniate larva (so far as is known), internuncial cells do not play ch an important role as they do in the early stages of the craniates. Floor- ate cells do not intervene in the sensori-motor arc as they do in the diagrams Conklin, and as we have seen above, the motor cells (which have not been entified) are almost certainly in direct contact axo-axonally with the Rohde lls. Only later, with the development of the A cells of Retzius, will inter- uncials be required in the system. At the stages examined, it is true that the cells in the extreme anterior end of the cord are not in direct connexion ith the presumed ventrally placed motor cells. We do not know enough bout the possible extent of the dendrite field of the motor cells to say if an ternuncial must intervene between them and the fibres of tract I. But dging by what is known about the relations of the dendrite fields of the ohde cells, it seems that these cells may also act an internuncial role, as ell as performing as primary sensory neurones in a monosynaptic sensori- otor arc. The B cells certainly send their fibres the length of tract I, into hich the dendrites of the Rohde cells ramify.

The suggestion is, therefore, that the Rohde cells play a more important le in the Acrania than do Rohon–Beard cells in the craniates. It is for this ason that I am reluctant to homologize them with the Rohon–Beard system. some ways, the A cells of Retzius would be more suitable candidates for e title of the homologue of that system in the Acrania. These cells are found the same position as Rohon–Beard cells, and their processes are apparently ranged in the same manner, i.e. to the periphery through dorsal roots, and teriorly and posteriorly in the dorsal columns of the cord. That they play more important role after metamorphosis than before it, might merely imply at the Rohon–Beard system develops more precociously in the craniates a result of the abandonment of the Rohde system. But such speculations e not profitable; the point is made in order to show that more information required before we can attempt to homologize the systems of cells in the crania with those characteristic of the craniates.

So far as I have been able to observe, there are not large, segmenta arranged somatic motor cells along the main part of the cord in the lar studied. As stated above, large cells are found at the caudal part of the co and they may perhaps be regarded as somatic motor cells; they are not fou anterior to the ilio-colonic ring. If this can be confirmed, then it is a striki difference from any craniate larva hitherto examined. Reference to Whitin figure of the arrangements in the pro-ammocoete larva (Whiting, 1955) w show how distinct these cells are in the craniate larva. I am almost convinc that if such large cells were to be found in the acraniate larva, then they wou have been visible in my preparations, and I incline to the view that soma motor cells are at this stage smaller than in the pro-ammocoete larva, an that they form a more dispersed system.

Whiting's view was that 'the ancestor of the vertebrate would posse numerous large and distinctive neurones, paired or unpaired, carrying o between them sensory, motor, correlating, and co-ordinating functions: su a team, measured in hundreds and not millions, might at first be the pr dominant part of the central nervous system' (Whiting, 1957).

Such large cells would act as 'focal points' collecting other cells performi the same functions round them, so that in brain nuclei, for example, as we as in the segmental patterns of the cord, there might be found a single lar cell surrounded by smaller cells performing the same functions, which ha come to operate around the centre provided by the large cell.

The condition of the somatic motor cells of the acraniate larva sugges that here either the large cells have disappeared before the larva reaches th stage examined (in the way that the large transient Rohon–Beard cells di appear in Amphibian ontogeny, for example), or that there are not larg cells of this kind in acraniate ontogeny.

That there are possible somatic motor cells in the caudal part of the cor suggests that they may have disappeared by the time the larva reached th stage where it is about to metamorphose.

Comparison of stages in ontogeny between the larvae of different grou is always difficult, but especially so in this case, for it is not at all simple t decide which stages are equivalent. It should be borne in mind that th acraniate larva hatches precociously as the late neurula, and is capable o swimming movements from a very early age. Moreover, the metamorphos of the acraniate is probably not homologous to that of the Agnatha, so tha it cannot well be used as a fixed point for comparison.

On the other hand, it is reasonable to suppose that the condition of th cord in the Acrania will prove to be more primitive than in any craniate, s that a late-stage acraniate larva would be equivalent to an early stage i craniate development. As at present we only know a little about a single stage i the development of the acraniate, it does not seem worth while to attempt an closer analysis of the differences between the acraniate and agnathan spinal cord When more is known of the other stages in acraniate development, it will b easier to see at which stage the larvae of the two groups should be compared

I should like to thank Dr. William Holmes for his constant encouragement. ave been much assisted by discussion with Dr. H. P. Whiting. Part of this rk was carried out in the Department of Zoology, Oxford, during the ure of a prize fellowship at Magdalen College.

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The Possible Calcification Mechanisms in some Reptilian Egg-shells

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SUMMARY

udies have been made of the organic matrix of certain reptilian egg-shells. The raction between egg-shell matrix and various metal ions has been considered by ng the effect of these ions on the staining of the matrix by toluidine blue. A com- son of the results with those for the hen indicates that the chelating mechanism in Chelonia is similar to that in the hen, but that that in the Crocodilia is different. suggested that in the Crocodilia the acid mucopolysaccharide of the matrix is edded in, but not combined with, the protein and that its chelating mechanism is oxylate group to carboxylate group, while in the hen and Chelonia, the acid opolysaccharide is combined with the protein and that its chelating mechanism is oxylate group to amino group.

INTRODUCTION

has been shown by Simkiss and Tyler (1957) that, in addition to protein, he organic matrix of the hen egg-shell contains an acid mucopoly- haride. Later work (Simkiss and Tyler, 1958) indicated that this acid opolysaccharide acted as a chelating agent binding calcium ions to the rix and thus influencing the later precipitation of the calcium carbonate in egg-shell. These experiments produced evidence that the electron donor ups, which form the chelate ring, involve carboxylate and amino groups. e results suggest that it might be possible to discover some chelate in an -shell matrix which could represent an ancestral type, in an evolutionary se, to the egg-shell matrix of the hen.

his paper describes the results obtained from some reptilian egg-shells by pplication of the methods used to study the organic matrix of the hen -shell. Throughout this paper the term 'carboxylate' is used to describe ionized acidic groups which are attached to the mucopolysaccharide. It is hly probable, however, that sulphate groups are also involved and this uld be borne in mind. The term carboxylate, therefore, is used as a simple convenient way of advancing the argument without constantly having to r to the two types of group and it has been preferred to sulphate because s simpler structure.

MATERIAL

gg-shells from the following reptiles were examined in detail: *Crocodilus sus*, *Caiman sclerops*, *Trionyx euphraticus*, *Testudo graeco*, and *Podocnemis eriliana*. *Testudo emys* was examined in less detail. None of this material

rterly Journal of Microscopical Science, Vol. 100, part 4, pp. 529–538, December, 1959.]

was fresh, but wide experience with many avian egg-shells indicates that organic matrix of the shell suffers no change if the shell itself is not erode

Throughout the paper the terminology will be that used by Simkiss a Tyler (1957) for the hen egg-shell, but this should not be taken to imply t the various regions of the reptilian egg-shell are necessarily homologous w those of the avian egg-shell.

METHODS

The material was decalcified and fixed in the way described by Simkiss a Tyler (1957). The decalcified material was embedded in wax and sectio were stained by Mallory's triple stain, mucicarmine, and the tetrazo- naphthol reaction. The methylene blue extinction (MBE) method was a used at pH 2·6, 3·2, 3·8, and 4·3, with M/2000 solutions of stain in barbito buffer (Pearse, 1953).

The elimination concentration, i.e. the concentration of metal ions requir to eliminate the metachromatic staining obtained with 0·01% toluidine blu was measured as described by Simkiss and Tyler (1958), by the use of different metal cations. In the case of the ferric ions, the influence of ti upon staining was also noted. So many staining experiments had to be do that they could not all be carried out at one time, nor could all concentratio be used. Therefore, the readings can only be regarded as approximate.

The method of Fisher and Lillie (1953) was used to study the effect methylation upon metachromatic staining.

RESULTS

The general structure of the reptilian egg-shells was shown with Mallor triple stain. The matrix stained blue and there was a red-staining membra as in the avian egg-shell, but there was no obvious cuticle in most specimen although an irregular layer is present in *Testudo emys*. On the inside of t shell there were structures rather similar in appearance to the mammilla knobs of the avian egg, but they are not necessarily identical in form function. These knobs did not seem to possess the distinctive cores usua seen in avian egg-shells. In most specimens, masses of matrix appeared form the connexion between the shell and the membrane and this correspon to the spherulite region described by Young (1950). This author also stat that there is a cuticle but no matrix in the egg-shell of *Testudo*, but this is co trary to our findings.

In all the reptilian egg-shells used, the tetrazo-β-naphthol and the muc carmine methods have shown respectively that proteins and acid muc polysaccharides were distributed evenly throughout the matrix. Methylati before staining with toluidine blue abolished metachromasia in all the reptili shells examined.

A few interesting general observations made during the experiments elimination concentration are best mentioned here. In the case of *Crocodil porosus* and *Caiman sclerops* it was seen that there was no metachromat ning of the matrix at the points where it was attached to the shell mem- es. This was found to be true even in the absence of any metal cations n the solution of toluidine blue. This is possibly owing to the reaction of acidic groups of the mucopolysaccharide with the amino groups of the c amino acids in the membrane. The metachromasia of the matrix from e two samples was also much redder than in the other reptilian or avian -shells which have been studied. Thus in pure toluidine blue solutions

TABLE 1

effect of time upon the elimination concentration with ferric ions for the Crocodilia

+ = positive; — = negative

Fe^{+++} *as* $FeCl_3$ *m. eq. per litre*	*Time* (*h*)	Crocodilus	Caiman
10	12	+	weak+
10	48	+	weak+
50	1	trace	trace
50	12	trace	trace
50	48	trace	trace
75	1	trace	trace
75	12	—	—
75	48	—	—
100	1	trace	trace
100	12	—	—

TABLE 2

Elimination concentrations measured in m. eq. per litre

Cation	*Hen*	Testudo	Trionyx	Podocnemis	Crocodilus	Caiman
$^{++}$ (as $FeCl_3$)	. .	. .	. .	. .	10	10
$^{+++}$ (as $Nd(NO_3)_3$)	4	<4	<4	<4	150	175
$^{+}$ (as $CuCl_2$)	5	2	5–10	10	300	300
$^{+}$ (as $NiCl_2$)	70	25	100	100	300	300
$^{+}$ (as $ZnSO_4$)	100	50	?	150	350	350
$^{++}$ (as $MnCl_2$)	160	50	150	160	350	350
$^{+}$ (as $CaCl_2$)	180	60	150	200	300	300
(as NaCl)	400	200	400	400	400	400

ocodilus porosus stained much more deeply than *Testudo graeco*, but this ference in intensity of staining does not, of course, interfere with the assess- nt of elimination concentrations.

Some difficulties which arose in the experiments on elimination con- tration must be considered. Preliminary experiments with aluminium ions l ferric ions on *Crocodilus porosus* and *Caiman sclerops* showed that at m. eq. per litre there was still a positive staining result after 2 h but that er 24 h it was lost. This suggested that these shells were taking a long time reach equilibrium. In the elimination concentration experiments proper, ric ions were therefore allowed 48 h to reach equilibrium and readings with all other ions were checked over at least 24 h to ensure that any alterat would be noticed.

The elimination concentration for ferric ions was taken as 10 m. eq. litre in the case of the caiman and crocodile. Some staining remained greater concentrations than this, but it was weak and irregular, and var with time, and was therefore ignored (table 1).

TABLE 3

Methylene blue extinction values

+ = positive; — = negative

pH	2·6	3·2	3·8	4·3
Crocodilus	+	+	+	+
Caiman	+	+	+	+
Podocnemis	—	—	—	+
Testudo	—	—	—	trace
Trionyx	—	—	—	trace

Trionyx euphraticus had a very sparse matrix on the inner, but a mu denser one on the outer layers of the shell. This denser portion may possi be a cuticle and therefore the elimination concentration for the inner port has been taken.

The results for all the experiments are given in table 2.

In the methylene blue extinction experiments the readings were taken af staining for 24 h at various pH levels. The results are shown in table 3.

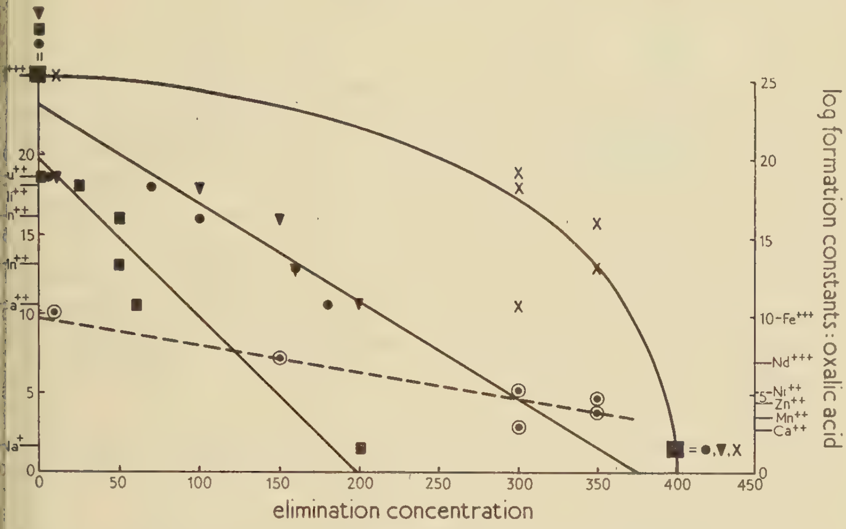
DISCUSSION

In the original paper describing the study of elimination concentrati (Simkiss and Tyler, 1958), experiments were performed to show that t elimination of staining from the shell matrix by various metal cations was due to any direct interaction of the stain with the metal. The present resu confirm the soundness of the method, for a section of a hen or chelonian e shell will not stain in a solution of 150 m. eq. per litre of copper ions in 0·01 toluidine blue, whereas the matrix of a crocodilian egg-shell will stain. T confirms that the results are determined by the specimens used and not interactions between cation and stain. Furthermore, the differences betw various shells are not simply matters of degree, as might be expected from differences in the original intensity of staining, but are basic differences the relative concentrations of cations required to eliminate metachrom staining.

The egg-shells of the 5 reptiles which have been studied in detail representative of two orders, namely, the order Chelonia, which includes sub-order Pleurodira or turtles (e.g. *Trionyx* and *Podocnemis*) and the s order Cryptodira or tortoises (e.g. *Testudo*), and the order Crocodilia (*Crocodilus* and *Caiman*).

The elimination concentration results in table 2 show that the values

Chelonia are all of roughly the same magnitude as those for the hen, and, her, the two pleurodiran specimens are almost identical with each other. se results are clearly seen in fig. 1 where the elimination concentrations been compared with the log. formation constants of a 'carboxylate-no' type of chelating agent, namely, ethylene diamine tetra-acetic acid TA). In all cases there is a strong correlation. The values for the hen



ɪ. Graphs showing the relationship between elimination concentration and log. formation constant.

Hen
Testudo
Podocnemis
Crocodilus
} Log. formation constants with EDTA

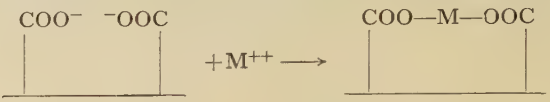
Crocodilus; log. formation constants with oxalic acid.

e lines are put in as guides and are not intended to indicate a mathematical relationship. avoid confusion, the results for *Trionyx* (similar to *Podocnemis*) and *Caiman* (similar to *odilus*) have not been included in the diagram.

ly results for metals with known log. formation constants could be included in the graph.

kiss and Tyler, 1958) have been given for comparison, and the values for turtles are very similar to them. On the other hand, the results for *Testudo* slightly different from those for the hen in position. This suggests that the shell matrices of the Chelonia show a similar chelating mechanism to TA and that, therefore, both amino and carboxylate groups are involved. ther, the chelating power for *Trionyx* and *Podocnemis* is about the same as he hen, but that for *Testudo* is somewhat stronger.

able 2 shows that the elimination concentrations for the Crocodilia are different from those for the Chelonia and the hen. Also, when they are pared with the log. formation constants of EDTA (fig. 1) there is no le linear relationship. The explanation of this difference may be seen in 2, if the bivalent metals only are considered. In the hen the range of values from copper to calcium covers 5 to 180 m. eq. per litre, but in t Crocodilia it only covers 300 to 350 m. eq. per litre. This similarity of acti of all the bivalent ions suggests a complex formation with one bivalent me ion, M^{++}, neutralizing two carboxylate groups as follows:



$$\text{COO}^- \quad {}^-\text{OOC} + \text{M}^{++} \longrightarrow \text{COO—M—OOC}$$

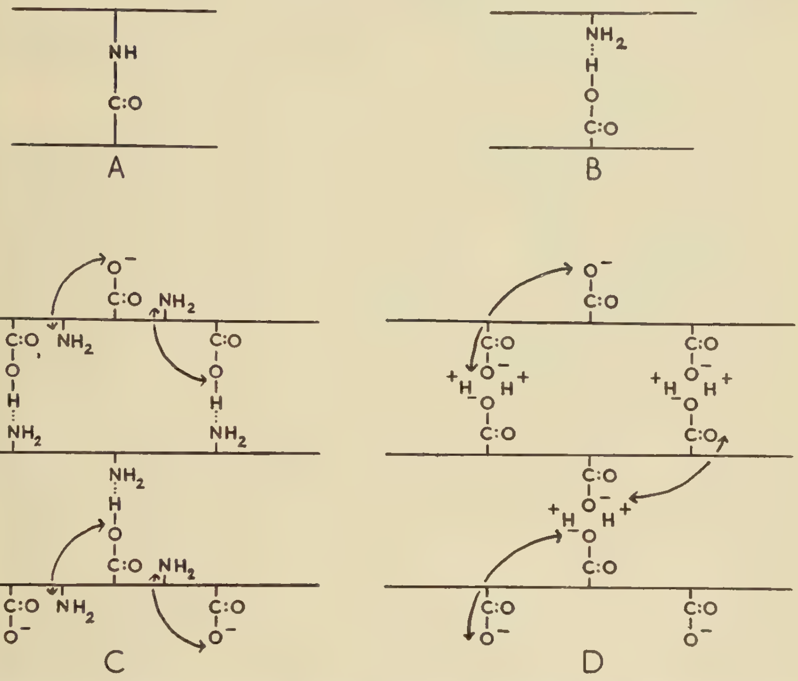
It is clear, however, that such a representation still indicates a form of chel tion but with two carboxylate groups instead of with one carboxylate and o amino group. The simplest model with which this chelation effect can compared is oxalic acid, and in fig. 1 this has been done. Unfortunately, the are no common metals with log. formation constants for oxalic acid betwe 6 and 10 and, therefore, the results are not very well spaced out. In order overcome this difficulty neodymium, with a log. formation constant of 7 with oxalic acid, has been used as an additional metal in the elimination co centration experiments. The results provide strong evidence (fig. 1) of a line relationship, which suggests that in the Crocodilia the egg-shell matrix ac as a dicarboxylate chelate.

In order to be able to understand the other results obtained, it is necessa to compare them with the hen egg-shell, which has been studied in mu greater detail.

Working in these laboratories, Baker (unpublished) has shown that the ac mucopolysaccharide in the hen egg-shell is chondroitin sulphuric acid. Th differs from the original suggestion of Simkiss and Tyler (1957), who co sidered, from histological work, that the matrix probably contained mucoit sulphuric acid. However, the difference in results is reconcilable. The origin histological work with hyaluronidase was performed upon sections embedd in wax (Pearse, 1953), and the matrix was found to be stable. It has now be shown (Lea and Vaughan, 1957) that paraffin embedding of the specim affects the action of hyaluronidase upon it, and when the experiment w repeated with frozen sections, it was discovered that the egg-shell matrix wa in fact, labile to the enzyme. This result thus makes the histological wo compatible with the chromatographic work of Baker, and stresses the need f a fuller investigation of the use of techniques involving enzymes.

The methylene blue extinction coefficient for the hen and for Cheloni specimens shows a value of pH 4. Since chondroitin sulphuric acid is no known to be present in the organic matrix of the hen egg-shell, it would appe that it is unlikely to be free, since, if it were, it would probably bind the sta at a much lower pH than 4. The acid mucopolysaccharide in the matrix of t Crocodilia need not necessarily be identical with that of the hen, but t MBE value of 2·6 suggests that whatever its detailed structure is, it exists a free state in the matrix. The tetrazo-β-naphthol reaction indicates that pr tein is distributed evenly throughout both the matrices. It appears, therefo t the acid mucopolysaccharide may be bound to this in the hen and in the elonia but not in the Crocodilia.

he mechanism of binding the acid mucopolysaccharide to the protein is bably by means of an attachment of the carboxylate groups of the muco- ysaccharide to the free amino groups of the protein (Meyer, Palmer, and yth, 1937). There are two possible mechanisms for this, namely, by a



2. Diagrammatic representation of the various kinds of chemical combinations discussed he text. A, peptide bond. B, hydrogen bond. C, pairs of chelating groups, i.e. carboxylate amino, are shown by arrows. The middle chain is a protein chain and the outer chains are ndroitin sulphuric acid. D, pairs of chelating groups, i.e. carboxylate and carboxylate, are wn by arrows. Only chondroitin sulphuric acid chains are shown, because the protein chains are not intimately bound with them.

tide linkage or by hydrogen bonding (fig. 2, A, B). However, since the boxylate groups of the mucopolysaccharide are also required as electron iors in chelation, it follows that hydrogen bonding is more likely than tide linkages. This concept is supported by the evidence of methylation. was found that methylation completely abolished metachromasia with uidine blue, and methylation is far more likely to be possible with a lrogen bond than with a peptide link. Of course, this argument assumes t the peptide link could induce metachromasia in the first place and, ording to most current theories of metachromasia, this seems unlikely, ess the toluidine blue cation actually enters into a chelate ring in its meta- omatic form. If this possibility is rejected and the peptide link is con- ered to be incapable of inducing metachromasia, this merely provides

further evidence for the absence of a peptide link, since all egg-shell matric
so far studied have stained metachromatically. This possibly also explai
why the region of attachment of the matrix to the membrane does not sta
metachromatically in the Crocodilia, where there are no mammillary co
and where it is suggested that the acid mucopolysaccharide of the matrix m
be attached to the shell membrane by a peptide link.

The effect of stretching on sections of decalcified avian egg-shells follow
by staining, has suggested that the protein / acid mucopolysaccharide linka
may be easily destroyed physically (Simkiss, 1958). This also tends to favo
the idea of hydrogen bonding rather than a peptide link.

Thus the evidence points to the fact that, in both the hen and Chelonia eg
shell matrices, chelation occurs between carboxylate and amino groups a
that the protein is hydrogen-bonded to the acid mucopolysaccharide. This
shown diagrammatically in fig. 2, c. In the Crocodilia a different type
matrix is found. Here the protein is not bound to the acid mucopolysacchari
and chelation is between two carboxylate groups (fig. 2, D). It is interesting
note that the physical effects which appear to break the protein / acid muc
polysaccharide linkage in hen egg-shells have no effect on the matrix of t
egg-shells of the Crocodilia.

The carboxylate-amino type is clearly a stronger chelate than the dicarb
xylate type. This is shown by the elimination concentrations for calciu
cations (table 2), which is the cation in the shell. The concentrations requir
to eliminate staining are about twice as large in the Crocodilia as in the Chelon
and the hen, which shows that the Crocodilia has only about half the chelati
power of the others.

It would appear that for shell formation, the chelating power of the eg
shell matrix must be strong enough to remove calcium ions from the bloo
but weak enough to be disrupted by carbonate ions, thus allowing the calciu
to precipitate as carbonate. Within these limits, the stronger the chelati
power, the greater will be the concentration of calcium ions achieved on t
forming egg-shell and the better the calcification. A chelating molecule in t
egg-shell matrix requires stabilizing and orientating if it is to function sati
factorily. Thus if the chelating molecule is likely to move towards the calciu
ions instead of the calcium ions coming to a fixed chelating molecule, a we
shell structure would result. This difficulty is overcome if the chelating mol
cule is embedded in a protein matrix, without any direct combination, an
this is possibly the structure in the Crocodilia. An alternative way is to atta
the chelating molecule to the protein and this could result in an orientat
molecule and hence a better chelate. This is possibly the situation with t
Chelonia and the hen.

Before we can try to understand these two systems and their relationshi
one other fact must be mentioned. It has been shown chromatographica
that the acid mucopolysaccharide of the crocodile egg-shell matrix actua
contains uronic acids and amino sugars, and hence carboxylate and ami
groups (Baker, unpublished), while the idea put forward above has been th

e matrix contains only carboxylate groups. There is, however, no anomaly re, for the work which has been reported in detail has been concerned with e functioning of the matrix and, far from confusing the case, the chemical mposition of the acid mucopolysaccharide clarifies the way in which the o matrix systems may be related from the point of view of evolution. A ore efficient matrix can be obtained from the dicarboxylate chelate of the ocodilian egg-shell without any break in the physiological functioning of the ructure, by the binding of the acid mucopolysaccharide on to the protein. his provides the possibility of orientation within the molecule so that the ructure can form a chelate ring between acid and amino groups, both of hich are apparently present in the Crocodilian egg-shell matrix, but which pear to be sterically incapable of forming a chelate ring without the orientan provided by the binding of protein. It is impossible to say, on the evidence ailable, whether the amino group which enters into the chelate ring resides the acid mucopolysaccharide or on the protein. However, the result of this nding is to produce a stronger chelating agent and a structurally sounder atrix such as appears to be found in the egg-shell of the hen and Chelonia.

The experiments which have been reported suggest two different types of lcifying mechanism which might be related in an evolutionary sense. From rather different point of view, it is interesting to mention some preliminary sts on bone. These results are not so easy to interpret, but it is evident that e medullary bone found in the marrow cavities of the bones of laying hens as a lower methylene blue extinction value (pH $< 3{\cdot}0$) than cortical bone MBE, pH 4). This suggests that the acid mucopolysaccharide in medullary one differs from that found in cortical bone in that it may not be bound to rotein. In this connexion, Taylor and Moore (1953, 1954) have shown that edullary bone acts as a labile store of calcium, which is used during shell rmation and deposited during the resting period between the formation of wo shells. On the above suggestion, medullary bone may have a similar type f matrix to crocodilian egg-shells, and cortical bone a similar type to the ther egg-shells. The poorer combining power of crocodilian matrix with ations and the greater lability of the medullary bone may, therefore, be imilar phenomena.

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The Histology and Activities of the Tube-feet of *Echinocyamus pusillus*

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With two plates (figs. 3 and 5)

SUMMARY

The histology of the suckered, buccal sensory, and respiratory tube-feet and their mpullae, where they occur, of the clypeasteroid sea-urchin *Echinocyamus pusillus* is escribed. Each suckered tube-foot possesses two sets of special muscles for attachment and detachment, a ring of mucous glands to assist in attachment, and a ring of ensory cilia. The stem retractors are in four columns, whose differential contraction rovides the means of postural movement relative to the test. The ampullae of these ube-feet are exceedingly thin-walled, apparently musculo-epithelial, with anastomosing contractile elements. The canal between tube-foot and ampulla contains a wollen coelomic epithelium which may help to maintain the nerve relationships of he system. The activity of the suckered tube-feet is compared with that of the tube-eet of the starfish, *Asterias rubens*. The buccal tube-feet, larger than the suckered ube-feet, have large disks underlain by a thick nerve plexus supported by transverse bres; a ring of sensory cilia surrounds the disk. They have no mucous glands and no uckers, and are presumably entirely sensory, probably both tactile (the cilia) and hemoreceptive (the disk). The respiratory tube-feet are thin-walled sacs, the walls onsisting of an outer ciliated and an inner non-ciliated (coelomic) epithelium with ross-connexions for support; where the coelomic epithelium lines the pair of canals hrough the test it is heavily ciliated. In the specializations of its tube-feet this urchin shown to share some features with the regular urchins and others with the spatan-oids.

INTRODUCTION

THE tiny irregular sea-urchin *Echinocyamus pusillus* (O. Fr. Müller) is the only member of the order Clypeasteroida found in European waters. ts normal size is 5 to 7 mm long and it seldom exceeds 10 mm. It normally ccurs in clean shell-gravel offshore, such as the Amphioxus grounds off lymouth, though it is known from coarse sand deposits between tide-marks, uch as the Black Rocks area of Anglesey. Mr. W. Galbraith tells me (personal ommunication, 1957) that while skin-diving off Majorca he noticed patches f shell-gravel interspersed in an otherwise sandy bottom; he found many pecimens of *E. pusillus*, and they were always confined to the shell-gravel egions.

In some features, such as the possession of a jaw apparatus, the clypeasteroids esemble regular sea-urchins, but in others, such as the specialization of the ube-feet and some features of their ciliary activity (Nichols, 1959*b*) they esemble the very specialized spatangoids, most of which burrow in the sea-ottom. *E. pusillus* also spends most of its time below the sea-bottom, though its normal substratum it cannot be said to burrow: more accurately it would

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be described as nestling in the interstices between the large particles wh pulling smaller particles on to its dorsal surface for further concealment. In sandy bottom, however, the urchin actively digs itself in, using the spines an tube-feet to shift material. In this it is unlike the burrowing spatangoi which use only the spines.

The purpose of this paper is to describe the histology of the suckere buccal, and respiratory tube-feet and their ampullae, where they occur, an to suggest their mode of operation. The activity of the suckered tube-feet shown to be similar to, though by no means identical with, that shown Smith (1947*b*) for the tube-feet of *Asterias rubens*.

MATERIAL AND METHODS

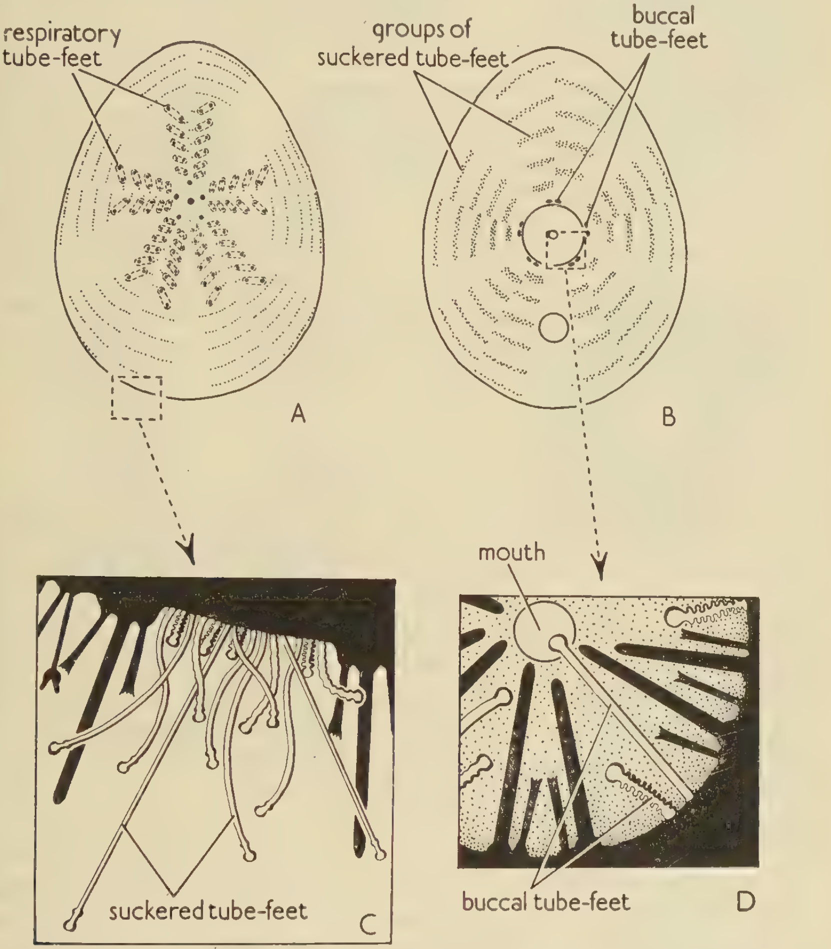
All the material used for histological study was obtained from the Edd stone shell-gravel, though some additional behavioural and morphologic observations were made on material obtained from the shell-gravel to t south-west of the Isle of Man. Whole animals were sectioned for histologic study. It is well known that clypeasteroids turn a bright green when injured seriously disturbed, and this showed which specimens should not be prepar for study. Both narcotized and unnarcotized material was used (see p. 54 Narcotization was effected by immersing the living animals in 2% solution benzamine hydrochloride in distilled water for 15 min. All specimens we fixed for 8 h, the fixative used almost exclusively being Heidenhain's 'Sus fluid made up in sea-water. The trichloracetic acid in this fluid successfull decalcified the specimens during the fixation time. The animals were th embedded by Péterfi's collodion/paraffin method (Péterfi, 1921), whi penetrates the very delicate tube-feet rather better than paraffin alone, a cut serially at 6 μ.

THE POSITION AND FUNCTIONS OF THE TUBE-FEET

The clypeasteroids have less specialization of the tube-feet than the spata goids, but more than the regular sea-urchins, the functional divisions *E. pusillus* (fig. 1) being as follows:

1. The suckered tube-feet, sometimes referred to as 'accessory' 'secondary' tube-feet. These are by far the most numerous, occurring groups of between 1 and 20 in association with every ambulacral plate. I ha been unable to see any line of demarcation between adjacent plates even sections, but Mortensen (1948) states that the tube-feet are borne in the sutur between the plates on the aboral surface (i.e. that their pores do not pier the plates themselves), but are not confined to the sutures on the oral surfac the groups spreading to each side of the sutures as well. Also, Mortensen sa that they are not confined to the ambulacral plates only but may occur also association with interambulacral plates. I have noticed that the suckered tub feet have two functions: first, they assist the large spines in locomotion, a secondly they pull small particles of the substratum on to the aboral surfa and help the spines to hold them there for concealment. When active, th

ave in an apparently random manner, a few only of each group extending at y one time. Even so, they sometimes jostle each other and cross stems while arching for a hard substratum. If they attach to a large particle they may sist in moving the animal, but if they touch a small grain, this may be lifted



IG. 1. Diagrams to show the position on the test and arrangement of the tube-feet of
. *pusillus*. A, aboral view of a test denuded of spines. The outlines of the respiratory tube-
et are dotted in round the pore-pairs. B, oral view of same. C, section through part of the
lge of the test of A, showing a single group of suckered tube-feet; the other appendages of
e test are shown in silhouette. D, part of the peristome, showing a pair of buccal tube-feet,
one fully extended; other appendages in silhouette.

nd passed in any direction, including up over the aboral surface. Subse- uently, another tube-foot may find the same grain lying against the test and ass it farther on. During these activities the tube-feet bend in an arc from eir bases, and do not hinge at their bases like a pendulum. This is important a consideration of the mode of operation of the muscle systems (see p. 549).

2. Buccal tube-feet. In each ambulacrum, where it borders the mout there is a pair of tube-feet which are slightly larger than the suckered tub feet. These appear to have their chief function during feeding, in explori the gravel particles for likely encrusting organic matter which can be rasp off by the teeth. Though they appear to be holding the particles steady duri feeding, in fact there is no sucker action; the disk merely rests against t particles.

3. Respiratory tube-feet. There are between 16 and 20 in the dorsal pai of each ambulacrum, in almost parallel double columns. Each tube-foot slightly flattened in one plane, corresponding to that joining the pair of por which pierce the test beneath them. These pores each carry a canal from t lumen of the tube-foot to its branch of the radial water-vascular canal. T ciliary currents of the dorsal surface of the animal, described fully elsewhe (Nichols, 1959*b*) are in an oral direction, and some pass down the longitudin axis of each ambulacrum; the tube-feet stand at an obtuse angle to this axis direct the currents across their respiratory surfaces. They are about 0·1 m long and about the same in height above the test.

The suckered tube-feet

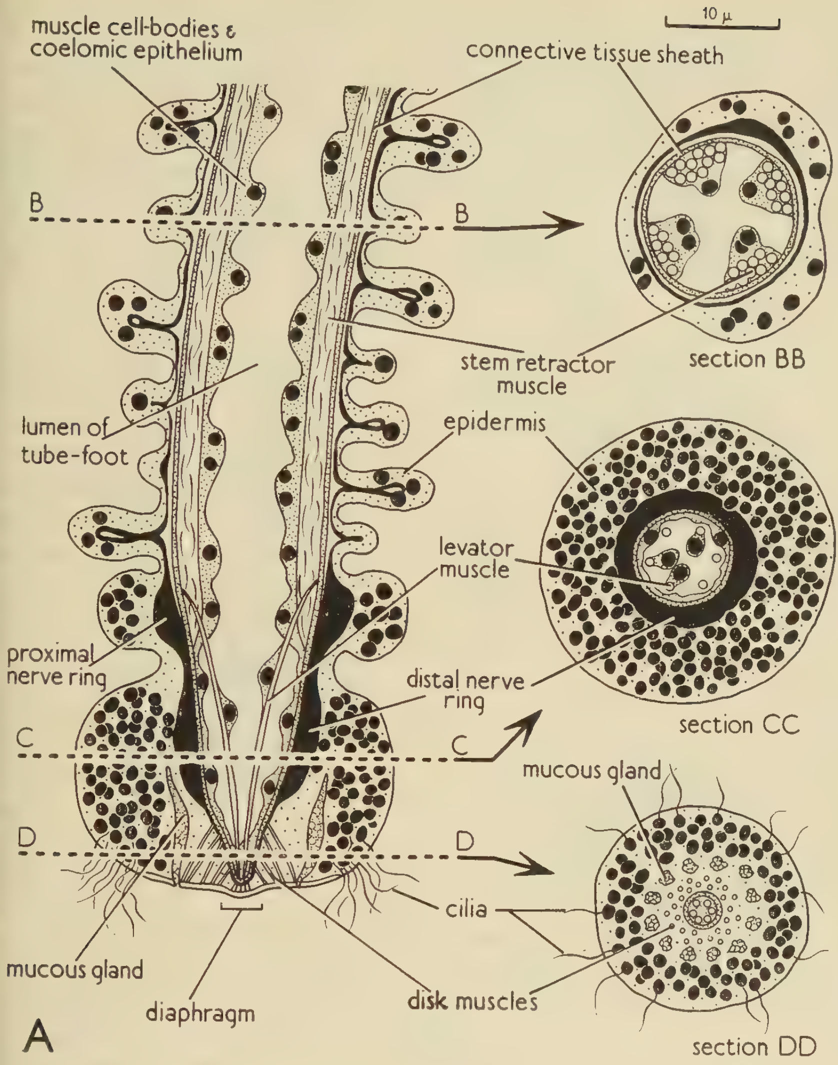
The relaxed length of these organs in narcotized specimens is seldom mo than 140 μ, and the width about 35 μ; they may extend up to 2 mm long, an when they do so the stem, like that of other tube-feet (see, for instance, Smit 1947*a*, p. 287), does not appear to alter in width to any extent, though becomes exceedingly thin-walled. When either retracted or protracted t end is in the form of a nearly spherical knob (fig. 2, A) and just proximal to th is another swelling, slightly narrower than the terminal knob. This swelling most prominent in the extended tube-foot; when retracted it is only slight more conspicuous than the folds of the stem epithelium.

The histology of the stem (figs. 2, 3). From the outside inwards the stem con sists of the following layers:

1. An epithelium, continuous with that covering the outside of the tes This is non-ciliated, some 3 μ thick with round or oval nuclei, 2 μ in diamete irregularly grouped in it. In sections both this layer and the nerve-lay immediately underlying it are found to have been thrown into folds, even narcotized animals, but in life these folds are taken up during protraction.

2. A sub-epidermal nerve plexus. This is seldom more than 1 μ thic except on one side of the stem (fig. 2, section BB), where it may thicken up 3 or 4 μ, and is the longitudinal tube-foot nerve. Since there is no cle demarcation between the epidermis and the plexus beneath it, it is sometim difficult to see this swelling in transverse sections. Between this layer and th next there is a space to permit extension.

3. A layer of connective tissue. This is 1 to 1·5 μ thick, and consists circularly-running fibres only. It provides the attachment surface for mar important structures in the tube-foot / ampulla system, and continues in



IG. 2. Diagrams to show the structure of an accessory tube-foot. The transverse sections on e right have been taken in the regions of the respective dotted lines BB, CC, and DD on the longitudinal section (A).

e interior of the test, where it is continuous with the much thinner conective tissue sheath of the ampulla.

4. The retractor muscles of the tube-foot. This layer is not continuous ound the whole stem but consists of 4 groups of fibres, each group being 3 to μ thick and made up of between 10 and 15 fibres to a group (fig. 2, section B). Each fibre runs the whole length of the tube-foot stem, being attached roximally to the connective tissue sheath lining the canal through the test nd distally to the continuation of this sheath in the tube-foot wall, in the

region of the swelling just proximal to the terminal knob. There are no nuc present in the region of the fibres, and there do not appear to be connexio between the sheath and the muscle-fibres elsewhere down their length. many transverse sections of the tube-foot stem (e.g. fig. 3, B) one of the fo groups of muscle-fibres takes up stain, such as the xylidine red of Panti (1945) modification of Masson's trichrome, to a greater extent than the othe In longitudinal sections too, the fibres on one side are often more heav stained than those on the other, and in this case it is always on the side t wards which a tube-foot is curved; this suggests that the heavily-stained fibr were contracting to a greater degree when fixed.

5. The innermost layer lining the lumen of the tube-foot. This varies thickness from about 1·5 μ to about 5 μ, and contains small round nuclei n more than 2 μ in diameter. In places, cell walls can be followed centrifugal between the individual fibres of the muscle-layer and seen to insert at t connective tissue sheath. This, together with the fact that there are no nuc in the region of the fibres themselves, suggests that this layer consists of t cell-bodies, with nuclei, of the muscle-cells, and a coelomic epithelium; th is, that the situation is similar to that in the spatangoid *Echinocardium cordatu* (Nichols, 1959*a*), in which the cell-bodies of the muscles bulge into the lum of the tube-foot. Distal to the region where the stem retractor muscles inser that is, in the terminal part of the foot, the innermost layer is very mu reduced in thickness, never exceeding 2·5 μ, and lies internal to the co nective tissue sheath; here the layer probably consists of a coelomic epitheliu only.

The histology of the terminal part. Towards the distal end of the tube-fo the connective tissue sheath constricts (figs. 2, A; 3, A) until, just proximal the disk, it encloses a cavity only about 2 μ in diameter. Within this co stricting part the only structures present, besides the very thin coelom epithelium, are 5 or 6 (generally 6) muscle-fibres arranged in a ring within t lumen of the foot (fig. 2, section CC). They are attached proximally to t connective tissue sheath close to the point where the stem retractors attac and distally to the inside of the connective tissue sheath at its most distal pa close to the disk itself. Each fibre has its own cell-body as a swelling som where down its length, containing the nucleus. This muscle system is close similar in structure and function (as is shown later, p. 553) to that of the levat muscles of the tube-feet of *A. rubens* (Smith, 1947*b*), so this term will be use for these also.

External to the connective tissue sheath down the length of the stem is space to allow the independent movement of inner and outer parts of t tube-feet during protraction. This cavity is not found in that part of the fo distal to the end of the stem retractor muscles; the sub-epidermal nerve plex lies against the sheath in the terminal part of the foot, swelling into a proxim and a distal nerve ring underlying the two terminal expansions of the ep thelium (fig. 2, A). With the techniques at present employed it is not possib to see whether the plexus extends round the distal end of the foot; by analo

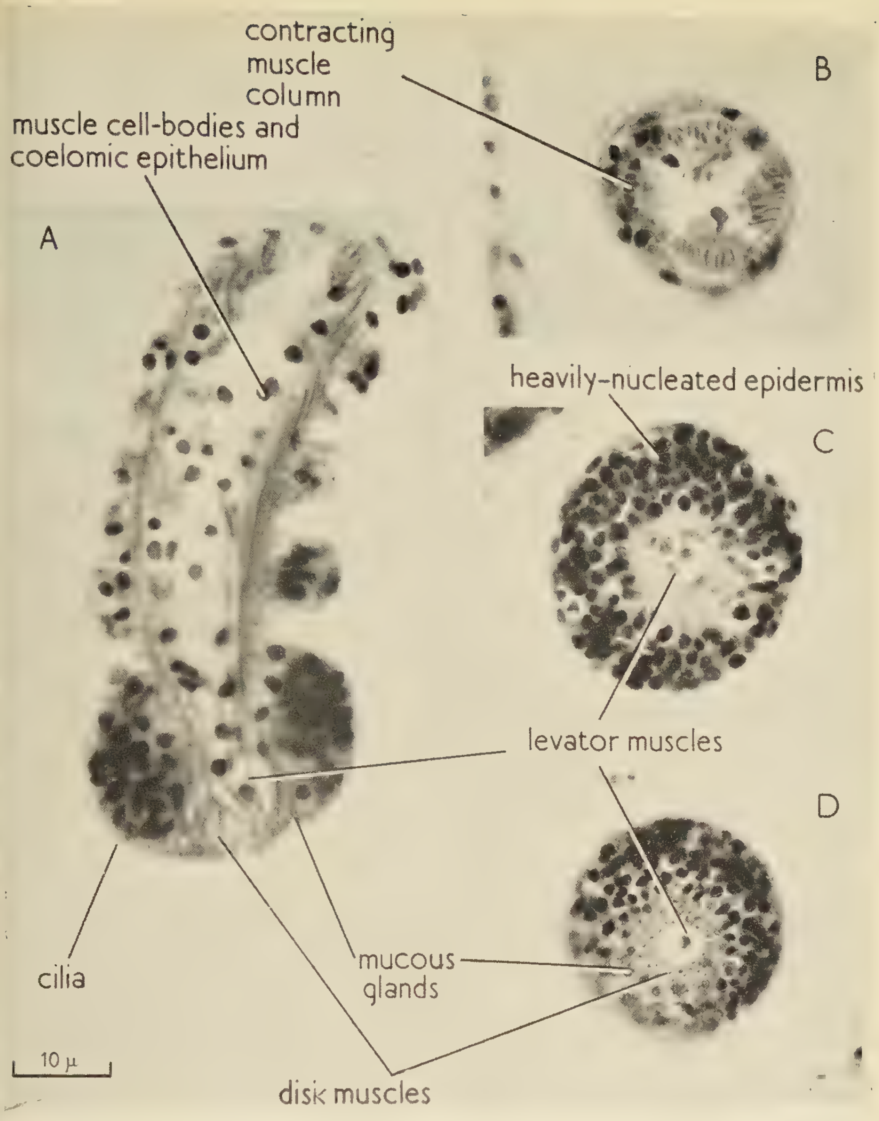

contracting muscle column
B
muscle cell-bodies and coelomic epithelium
A
heavily-nucleated epidermis
C
levator muscles
D
cilia
mucous glands
10 µ
disk muscles

FIG. 3

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FIG. 3 (plate). Photomicrographs of the same regions of an accessory tube-foot as are illustrated diagrammatically in fig. 2. All sections are of narcotized material, unless otherwise stated, and stained with Masson's trichrome as modified by Pantin (1948).

A, longitudinal section, approximately median. The section has passed through a column of retractor muscles on the right side of the stem, but not on the left.

B, transverse section of the stem. The specimen was apparently fixed while one of the columns of muscle-fibres was differentially contracting.

C, transverse section across the widest part of the terminal knob. Sections of the 6 levator muscles, some with their nuclei, can be seen in the central part, surrounded by the connective tissue sheath and distal nerve plexus.

D, transverse section across the distal part of the terminal knob, showing the levator muscles, much closer together than in C, the disk-muscles in section, and the ring of mucous glands, which appear slightly less dense than the nuclei of the epidermis.

th other echinoderm tube-feet (Hamann, 1887; Smith, 1937; Nichols, 59*a*) it seems likely that it does, passing between the bases of the disk-uscles.

Another set of muscles, the disk-muscles, is attached to the outer side of e connective tissue sheath proximally, and to the heavily cuticularized disk stally (figs. 2, A; 3, A). There are usually between 30 and 40 of these, each to 1 μ thick and varying in length from 2 μ in those towards the centre to μ in those towards the outside. They are apparently absent from the centre the disk (the diaphragm) and from the outer part of it.

Opening on to the disk in a circle roughly corresponding to the distal tachment of the outermost disk-muscles is a ring of 12 to 15 mucous glands g. 2, section DD), which may be up to 3 μ thick and 10 μ in length. They ain a vivid red in a Masson preparation, the contents appearing as globules out ½ μ in diameter. They do not stain with Southgate's mucicarmine, and hibit only faint γ-meta chromasia with toluidine blue or thionine.

Outside the heavily-cuticularized disk a large number of long cilia (10 μ so) protrude from the epithelium (fig. 2, A; section DD). These can be llowed into the swollen and heavily-nucleated epithelium of the terminal iob, and seen to arise from some of the nuclei there. This nucleated region also likely to contain the cell-bodies and nuclei of both the disk-muscles and e mucous glands, since there are no nuclei in the region of the disk-muscles on the centripetal side of the mucous glands.

The centre of the disk (the diaphragm) is separated from the outer side of e connective tissue sheath by 1 μ or so of epithelium. There appear to be ores stretching across this space, which could be either non-elastic or uscular. They are not continuations of the levators, because the sheath is ntinuous at their distal ends; they are unlikely to be more disk-muscles, ecause there is a definite region of undifferentiated epithelium between the itermost of them and the innermost disk-muscles. It seems most likely, erefore, from a functional point of view (see p. 547 below) that they are on-elastic fibres whose function is to convey the contractions of the levator uscles to the surface of the diaphragm.

The ampullae of the suckered tube-feet. In sections of narcotized animals it oved difficult to identify the ampullae with certainty, and impossible to escribe them adequately; in such sections they resemble flattened blisters the epithelium of the perivisceral coelom. In unnarcotized material the npullae were more distended. These observations suggest that when un-arcotized material was used, the action of the fixative caused all the muscles the system to contract. This had the effect of blowing up the ampulla vity with coelomic fluid during killing, at the expense of the lumen of the be-foot, the retractor system being the stronger.

Even when the ampullae are blown up in this way, the structure of their alls is not easy to distinguish. All that can be seen in section (figs. 4; 5, D) a very thin wall, not more than 1 μ and usually about ½ μ thick, with slight vellings at intervals containing the nuclei, about 2 to 3 μ long and 1½ μ wide.

The wall is not flat but thrown into folds every 2 μ or so, which in cros section are seen to be slightly denser on the outside of the ampulla. Pr sumably the dense part of these folds are the contractile strands of t protractor system, though in no sections did these structures take up stai normally taken up by muscle-fibres, such as xylidine red. These observatio suggest that the wall of the ampulla is a simple musculo-epithelial tissue;

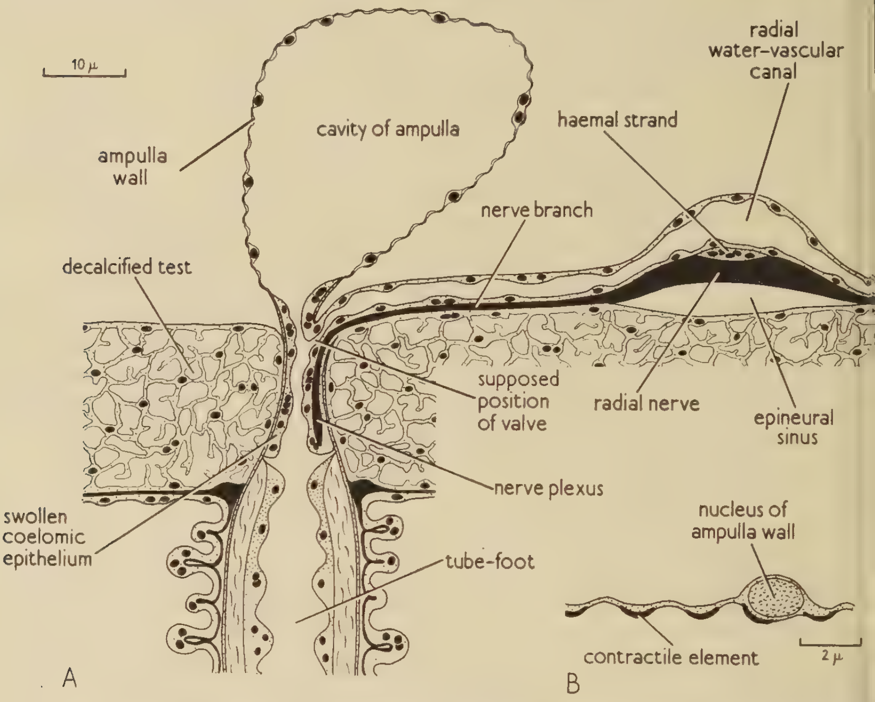

FIG. 4. A, diagram of the base of an accessory tube-foot, its ampulla, and its connexion wi the radial water-vascular canal and nerve-cord. B, enlarged view of part of the ampulla wa

basement membrane is visible, though the wall is too thin to be able to sa categorically that this more usual arrangement is not present.

The canals of the suckered tube-foot / ampulla system. Each suckered tub foot is borne by a canal which pierces the test and leads to the ampulla in th

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FIG. 5 (plate). A, longitudinal section of a buccal tube-foot showing the inner part of t sensory disk with its supporting fibres and the outer heavily-nucleated ring of ciliated cel the cilia cannot be seen in this section. Unnarcotized.

B, cross-section of a respiratory tube-foot, showing two of the cross-connexions of t coelomic epithelium, one slightly out of focus.

C, vertical section through a single respiratory tube-foot, showing the close mosaic of der nuclei of ciliated cells which line the canals through the test.

D, section of a single ampulla of an accessory tube-foot. Unnarcotized. Part of the swoll coelomic epithelium can be seen at the neck of the ampulla, where also one or two coelon corpuscles have lodged.

E, tangential view of the wall of the ampulla of a buccal tube-foot. Unnarcotized. On t lower right side part of the wall has been sectioned.

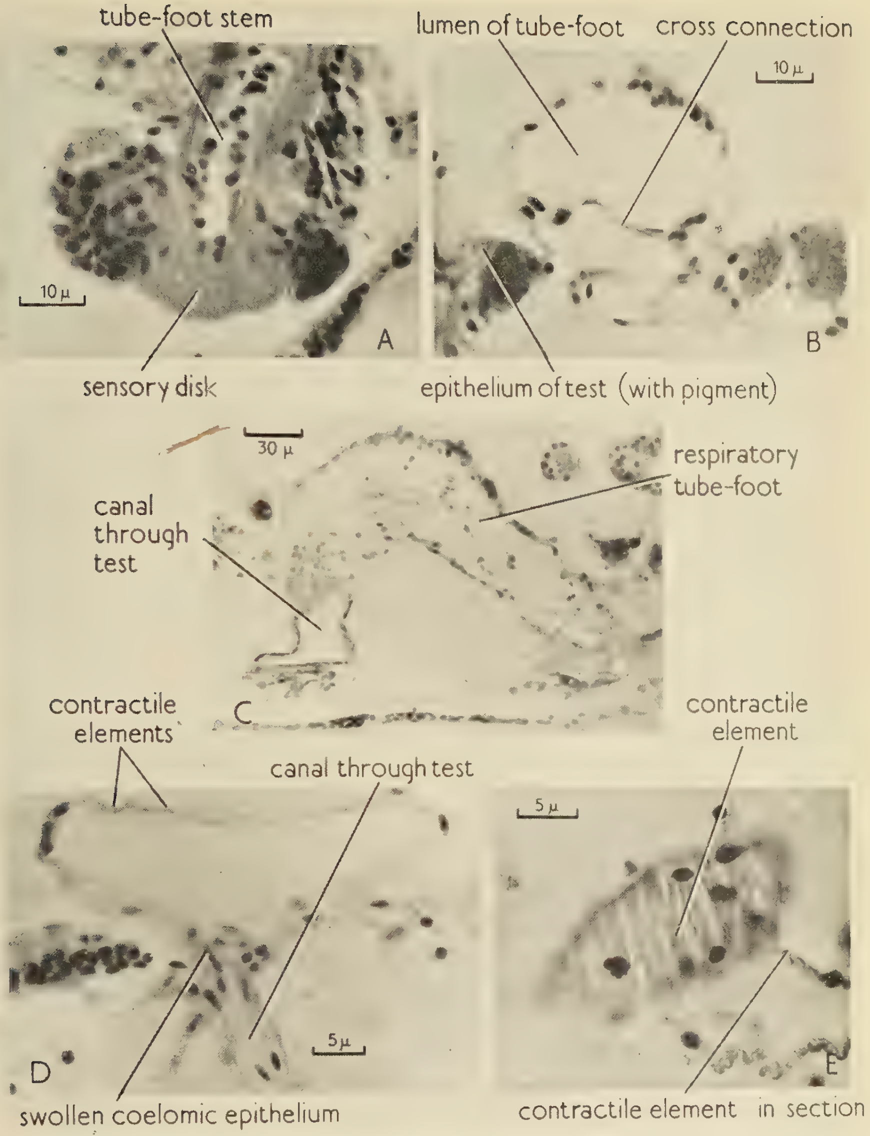

FIG. 5

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erivisceral coelom and to the radial water-vascular canal (fig. 4). Each tube-ot of a group is supplied by a separate lateral branch of the radial canal, hich may therefore give off anything up to 20 branches in the region of each nbulacral plate. The radial canal and its branches are lined by a coelomic oithelium which is quite thin (1 μ) along most of its length, except for slight vellings for the nuclei and embedded coelomocytes, but which swells up to μ or so where the lateral branch meets the tube-foot / ampulla canal. Both ange (1876) and Smith (1947*a*) describe valves at the junction of the lateral ranch of the radial water-vascular canal and the tube-foot / ampulla canal asteroids, and Smith (1947*a*) has also shown the necessity of such a valve r the efficient operation of each tube-foot. By analogy it is likely that a milar valve is present in each tube-foot / ampulla system of *Echinocyamus*; e slight swelling at the junction of the branch of the radial water-vascular nal and the tube-foot / ampulla canal may well constitute such a valve. No uscles have been detected in this area; but these would hardly be necessary, the flap over the exit of the lateral branch would certainly isolate the stem as soon as fluid started to flow from the ampulla.

The swollen epithelium continues into the canal to the point where the em retractor muscles arise. Similarly, the radial nerve gives off a branch to ch accessory tube-foot, and this passes into the enlarged epithelium of the be-foot / ampulla canal, where the nerve also enlarges and continues almost the point where the stem retractors arise. Smith (1950) describes what he lls bulbous masses at the junction of tube-foot and ampulla in *Astropecten regularis*, and ascribes to them the function of preserving the relationships the internuncial neurones in this area under the conditions of fluid flow sociated with the movements of foot and ampulla. These very much smaller d less pronounced structures in the tube-feet of *Echinocyamus*, with their mplement of nervous tissue, may well serve a similar function. I cannot see my preparations whether there is a connexion between the nerve plexus in is swollen coelomic epithelium and the sub-epithelial plexus of the suckered be-foot, though such a connexion certainly exists in the buccal tube-feet ee p. 551) where the nerve tracts are much more conspicuous.

Suggested mode of operation of the suckered tube-feet. The arrangement of e muscles in the tube-foot / ampulla system and the observed behaviour of e system suggest that the mode of adhesion is as follows.

As the tube-foot is protracted by the contraction of the ampulla muscles, e sensory cilia round the edge of the disk make the first contact with the bstratum (fig. 6, A). It seems likely that the resultant sensory impulse is nveyed first to the levator muscles, though with the techniques employed this work it is not possible to detect any direct connexion between cilia and uscles; the fact that the levator muscles attach to the stem connective tissue ose to that nerve-ring lying just proximal to the disk may be significant in is respect. Thus, when or soon after the distal face of the disk touches the bstratum, the levator muscles will be contracting and will raise the diaphragm the central part of the disk (fig. 6, C). At the same time, the mucous glands

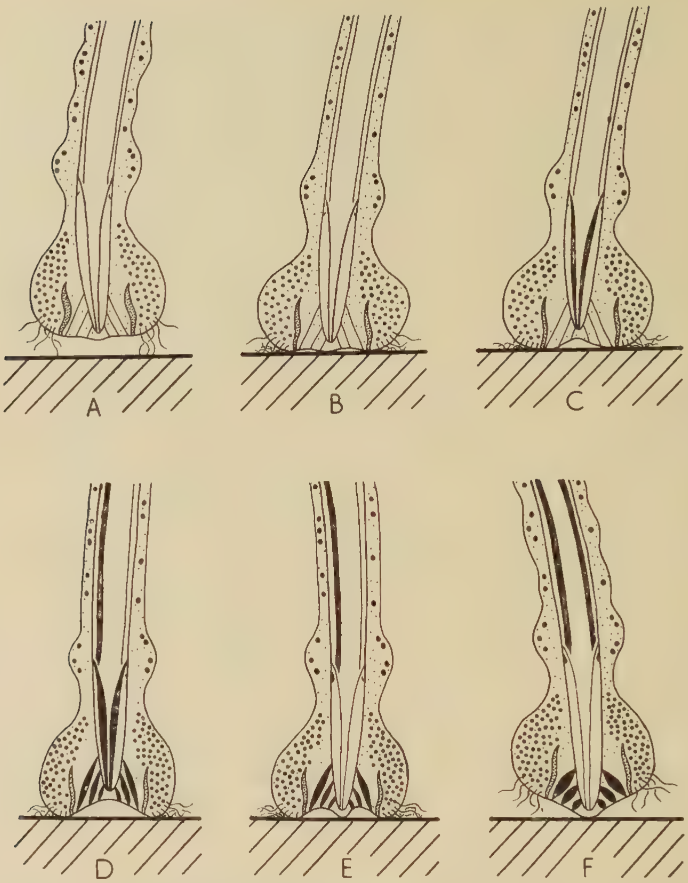

FIG. 6. Diagrams to illustrate the suggested activity of the sucker of an accessory tube
during adhesion. A, the tube-foot is being extended towards the substratum by the hyd
action of the ampulla (not shown), whose muscles are contracting. Some sensory cilia c
circle on the terminal knob are touching the substratum; none of the muscles of the tube
are contracting yet. B, the disk has reached the substratum; the mucous glands have sm
a sticky ring round the edge of the disk. C, the levator muscles are contracting and drawi
the centre of the disk (the diaphragm). D, contraction of the disk-muscles now enlarge
suction cavity while the levator muscles are still contracting. During this phase the postu
the tube-foot will be altered by contraction of one of the columns of stem retractor mu
moving the base of the tube-foot relatively to the point of attachment. E, the start of de
ment. The levator muscles relax but the disk-muscles continue contracting, causin
diaphragm to drop to the substratum. F, further contraction of the disk-muscles drav
the edges of the disk, so that the only part still touching the substratum is the diaph
The tube-foot is now withdrawn by contraction of the rest of the stem retractors, whi
ampulla muscles relax.

the disk will have exuded a ring of mucus on the outside edge of the disk d this will help both by its stickiness and in making a good contact for the cker to operate. Next, the disk-muscles will contract, and this will cause e rest of the disk to be raised away from the substratum, and will increase e volume of the suction cavity under the disk (fig. 6, D). Adhesion is now mplete, and the tube-foot can be moved relatively to the test.

The arrangement of the stem retractor muscles and their appearance in stological preparations suggest that any one of the 4 bands can be con-cted differentially. This activity will cause the whole tube-foot to bend an arc as has been observed (p. 541) and not merely from its base as if nged; in this respect it differs from the situation in some other echinoderms . 553).

After postural movement the disk will be ready to detach. For this, all that necessary is for the levator muscles to relax (fig. 6, E) while the disk-uscles still remain contracted. When this happens the diaphragm will drop d the disk edges will be pulled upwards, thus helping to unstick the mucous ng and raise the outer zone of the disk relatively to the centre (fig. 6, F). So e disk-muscles have a dual function: they help to enlarge the suction cavity uring adhesion and they are the active muscles during the act of detachment. ny of the other stem retractor muscle-bands can now contract, according to e new posture to be taken up, and the tube-foot can be swung to a new sition.

Further evidence in support of the suggested mode of attachment is pro-ded by allowing a tube-foot to attach to a coverslip or other thin plate of ass, and observing the sequence of events under the disk from the other side the glass. If the angle of the glass is adjusted relatively to the observer, it is ite easy to see when any part of the disk makes contact. The first part to uch is usually a point between the centre and edge of the diaphragm, and e area of contact spreads round in a circle, coinciding roughly with the rcle of pores from the mucous glands. Meanwhile, the centre of the disk has most always been raised by now, and the circle of contact moves outwards, . the suction cavity enlarges until the circle of contact corresponds with the dge of the heavily cuticularized part of the disk. On detachment, the dia-hragm drops on to the substratum first, then the edge of the disk is pulled wards and breaks contact, and finally the diaphragm is pulled clear.

The histology of the buccal tube-feet

The histology of the stem (figs. 5, A; 7) is similar to that of the accessory be-feet, so that a detailed description is unnecessary. Apart from slight dif-rences in the thicknesses of the various layers, the main points of difference ncern the connective tissue sheath and the muscle-layer. Towards the distal d of the stem, the connective tissue sheath consists of two layers: an outer yer with the fibres arranged longitudinally, and an inner layer with circular res. The outer layer is continuous with the longitudinal fibres of the sheath the terminal knob (see later) but is much thinner (about $\frac{1}{2}$ μ) in the region

of the stem retractor muscles, and thins out even more, possibly disappear
altogether, in the proximal part of the stem. The inner, circular layer is 1
2 μ thick, and stops abruptly at the point where the stem retractors ins
distally. Internal to this is the layer of stem retractor muscle-fibres. Thou
this may appear similar in longitudinal section to that of the accessory tu
foot, it is not broken up into quadrants but consists of a complete cylinder

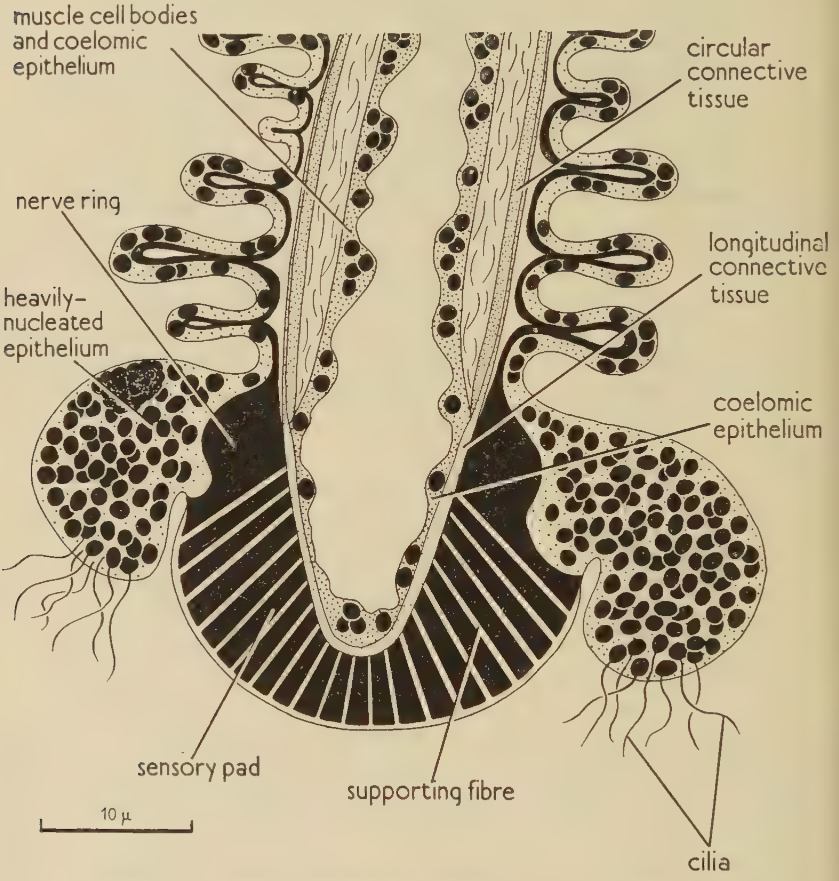
muscle cell bodies and coelomic epithelium
circular connective tissue
nerve ring
longitudinal connective tissue
heavily-nucleated epithelium
coelomic epithelium
sensory pad
supporting fibre
10 μ
cilia

Fig. 7. Diagram of a longitudinal section through a buccal tube-foot. Compare fig. 5,

Distally, as in other tube-feet, the sub-epidermal nerve plexus swells i
a nerve-ring. This occurs where the epithelium also swells to become
sides of the terminal knob; hence this nerve-ring is comparable with the dis
ring of the accessory tube-foot. The nerve-tissue underlying the disk its
expands considerably, and is traversed by many connective tissue-fibr
arranged somewhat radially (figs. 5, A; 7), inserted proximally into the co
nective tissue sheath, which here consists of longitudinal fibres only, a
distally into the thickened cuticle of the disk. There do not appear to be a

cous glands in the disk epithelium, and there are no muscles present in the
minal knob. The swollen epithelium of the terminal knob forms a ring
nd the outside of the disk; it contains many nuclei, as does the similar
elling of the accessory tube-foot, and often there are large aggregations of
ment, particularly towards the widest part. A ring of cilia is present in a
ilar position to the ring on the disk of the accessory tube-feet. The histo-
y thus supports the observation (p. 542 above) that the buccal tube-feet
not suckered but are totally sensory in nature; further to this, the ring of
a, common to both types of extensile tube-feet in this animal, and to tube-
t of other echinoids as well, e.g. *Echinus* (Nichols, in preparation), is most
ely tactile, and so very probably the vast expansion of the sub-epithelial
xus in the region of the disk is chemoreceptive. This suggestion has also
en applied to the buccal tube-feet of regular echinoids (Hyman, 1954).

The structure of the ampullae of these tube-feet (fig. 5, E) is very similar to
t described for the accessory ampullae. The walls, however, are slightly
cker and the contractile elements more conspicuous. The width of these
ments is about twice that of the elements in the accessory ampullae, and
quent anastomoses can be seen between adjacent elements.

The buccal tube-foot / ampulla system does not connect to a radial water-
cular canal but to the circumoral water-vascular ring. Similarly, the nerve-
xus lying in association with the swollen coelomic epithelium lining the
al through the test, which these tube-feet possess in common with the
essories, connects directly with the circumoral nerve-ring. The nervous
ue associated with the canal is much more conspicuous than that in the
ne situation in the accessory tube-feet, and it is possible to see that there is
onnexion here between the plexus of the coelomic epithelium and the ecto-
mal plexus of the tube-foot.

The histology of the respiratory tube-feet

These non-extensile tube-feet are stretched between a pair of pores, the
als both opening to one branch of the radial water-vascular canal. The
lls (figs. 5, B, C; 8) are 1 to 3 μ thick, depending on the presence of nuclei,
l composed of two layers. The outermost layer, as in other tube-feet, is
tinuous with the epithelium covering the test; on the dorsal surface of the
mal this layer may contain many large aggregations of pigment, though
se are never present in the epithelium of the respiratory tube-feet. The
at majority of nuclei in this layer belong to ciliated cells. These tend to lie
irregular patches, particularly on the sides. The cilia, seldom more than
r 4 μ long, have distinct basal granules in their cells. In the regions where
bands or patches are absent the outer layer is very much thinner (about
), though occasionally smaller nuclei occur which belong to undifferentiated
thelial cells. The inner layer of the wall consists of a coelomic epithelium
ut 1 to 2 μ in thickness. The nuclei of this layer are slightly smaller than
st of those in the outer layer, and belong to non-ciliated cells. An interest-
feature of these tube-feet is the presence at intervals of strands of tissue

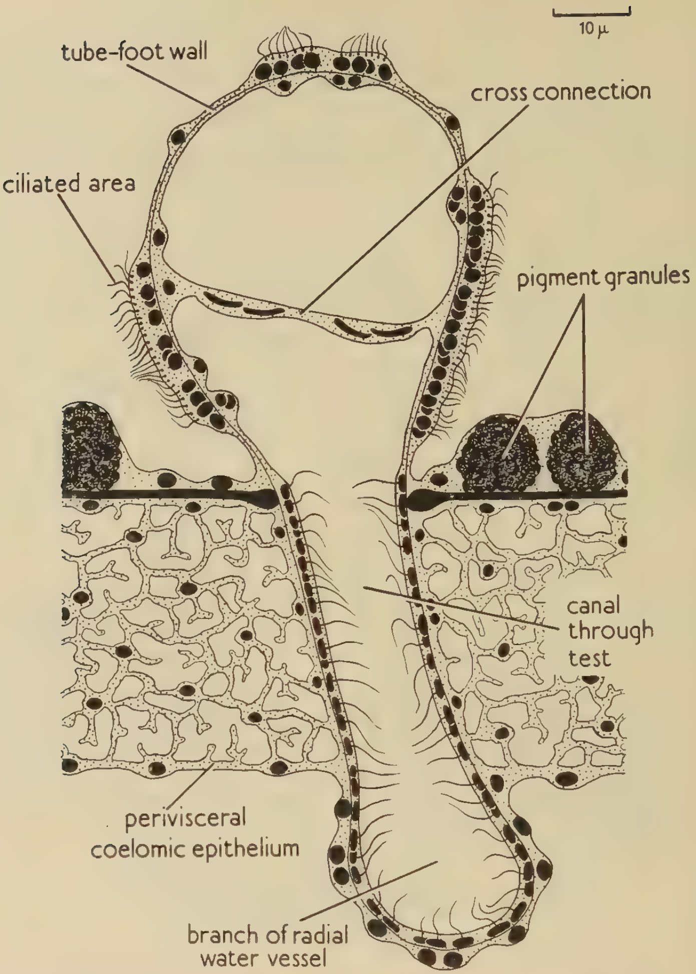

FIG. 8. Diagram of a transverse section across a respiratory tube-foot, showing the c through the test from the branch of the radial water-vascular canal, parts of three cili patches on the external epithelium of the foot, and one cross-connexion of the coel epithelium.

connecting across from one side to the other, continuous with the coelo epithelium. These contain flattened nuclei which appear slightly arcuat fixed preparations. There may be between 4 and 8 cross-connexions in a tu foot, and several may be seen at once in a transverse section (two in fig. 5

The canals through the test are lined with coelomic epithelium which c tains unusual horseshoe-shaped nuclei. In the centre of each of these nu is a conspicuous basal granule giving rise to a cilium of from 5 to 8 μ in len The branch of the water-vascular canal to each tube-foot is also lined intern by cells with horseshoe-shaped nuclei and large cilia, and is covered extern by a continuation of the perivisceral coelomic epithelium.

DISCUSSION

Smith (1947*b*) has produced the only account of the kinematics of echino- rm tube-foot activity. He has shown that in the type he studied, *Asterias ens*, there are four sets of muscles in the tube-foot / ampulla system: the otractors in the ampulla, the retractors in the tube-foot stem, the levator uscles for raising the centre of the diaphragm during adhesion, and the dial muscles of the disk for detachment. He also postulates a fifth set, the stural muscles, which encircle the base of the tube-foot stem to effect point- g, that is, to pull the longitudinal axis of the tube-foot away from the per- ndicular in relation to the arm. Smith gives reasons why he believes this parate set to be present, though he has not distinguished them anatomically *Asterias*; in *Astropecten*, however, they have been detected (Smith, 1950).

The main similarity between the systems of disk adhesion in *Asterias* and *Echinocyamus* is that both raise the centre of the disk by special levator uscles, though there is a slight difference in that some of those of *Asterias* iginate at the base of the tube-foot (Smith, 1947*b*, fig. 1), whereas in *Echino- amus* the levator muscles always attach to the connective tissue sheath wards the distal end of the foot.

The main differences between the systems are as follows. First, the muscles ncerned with detachment in *Echinocyamus*, the disk-muscles, lie on the stal side of the connective tissue sheath of the tube-foot, whereas in *Asterias* e analogous muscles, the radials, lie inside the sheath. Further to this, in *terias* they act in pulling down the centre of the disk (the diaphragm), hereas in *Echinocyamus* they pull the edge of the disk away from the sub- ratum. Secondly, the elaborate arborization of the disk connective tissue, hich in *Asterias* tube-feet conveys the pull from the levator muscles to the hole attachment surface of the disk, is reduced to the very short fibres in the ntre of the disk only of those of *Echinocyamus*. Such a system of fibres is deed unnecessary in the more centripetal part of the sucker in *Echinocyamus* be-feet, where they are functionally replaced by the disk-muscles.

The histological differences between the accessory and buccal tube-feet ave been dealt with (p. 549). One of these is the presence in the former of a elling proximal to the terminal knob, housing a proximal nerve-ring and n enlarged external epithelium with many nuclei. Internally, this is the point here the levator muscles are inserted proximally. This arrangement is con- stent with the suggestion that the ring of cilia at the disk, the nervous pansions of the distal and proximal nerve-rings, and the levator muscles rm a sensory-motor reflex arc.

The connexion between the sub-epidermal plexus of the buccal tube-foot d the circumoral nerve-ring is interesting. Presumably the buccal tube-feet, llecting both tactile and chemo-receptive sensory impulses, will operate in sociation with the jaw apparatus in selecting and dealing with food. The me functional connexion between sub-epithelial plexus and radial nerve- rd cannot be stated categorically in the case of the accessory tube-feet.

Circulation within the respiratory tube-feet is of more importance than in the others, and the provision of two pores and a one-way flow through lumen helps this. In addition, a tall flat structure will be more efficient gaseous interchange than a short thick one, and maintenance of this shap ensured by the presence of cross-connectives in the coelomic epitheliu without them it would be difficult to prevent the partial collapse of suc thin-walled structure. Finally, as well as internal circulation it is importa that sea-water in the immediate neighbourhood of the respiratory surfa should be kept moving. Since the animal may not be in a closely confin burrow but merely nestling in the interstices of a large-particled substratu a system of pumps in special areas of the test, like the fascioles of spatangoi would not be effective. Local currents are therefore produced by means ciliated patches on the external epithelium.

It has been mentioned above (p. 539) that the clypeasteroids share certa features with both the regular echinoids and the very specialized spatangoi Their complement of tube-feet further emphasizes their intermediate positi Most regular urchins possess special sensory buccal tube-feet on the peristo for use during feeding, and multi-purpose suckered tube-feet elsewhe Towards the dorsal surface in some regular urchins these tube-feet lose th suckers, apparently having only respiratory and sensory functions. In t spatangoids the specialization is more complete. Here, none of the tube-fe are suckered; some on the dorsal surface take on a solely respiratory functio and the rest are either mucous-producing organs or sensory feelers or bot The clypeasteroids share with the regular urchins suckered tube-feet over m of the surface and special sensory tube-feet for feeding round the mouth, a with the spatangoids some on the dorsal surface solely for respiration. T much phylogenetic importance should not be placed on similarities and di ferences in the tube-feet, however, since these structures in the same regi of quite closely related forms may have totally different functions; for exampl the adapical part of the anterior ambulacrum of the spatangid *Echinocardiu cordatum* bears highly extensile mucus-secreting tube-feet for burro building, whereas the same region of *Spatangus purpureus*, also a spatangi bears very much reduced tube-feet whose function appears to be whol sensory.

It is a pleasure to record my indebtedness to the Directors and Staff of t Marine Biological Stations at Plymouth and Port Erin for their assistance the collection of material; to Dr. A. J. Cain and Mr. Duncan Heddle for mo useful discussions in connexion with the work; and to Mr. J. Haywood f assistance with the photomicrography. Also I wish to thank Prof. Sir Alist Hardy, F.R.S., Linacre Professor of Zoology, University of Oxford, in who Department much of the work was carried out.

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Spicule Growth Rates in *Leucosolenia variabilis*

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With two plates (figs. 3 and 4)

SUMMARY

The growth rates of spicules of *Leucosolenia variabilis* have been determined by otographing stretched pieces of the wall for a maximum period of 17 h. For 3 pieces, B, and C, the average rates for spicule rays above 25 μ in length were respectively o (18–21° C), 1·64 (18° C), and 1·29 (17° C) μ per h. The discrepancies are greater n would be expected from the differences in temperature.

For both basal and oscular rays the growth rate increases to a steady level as the ys lengthen. The basal rays grow precociously at first, but in A the oscular rays soon ught up with the associated basal ray, while in B and C (both derived from the same cular tube) the oscular rays of the growing spicules tended to remain smaller than e associated basal ray during the recorded period of growth. Whether this difference tween the pieces was due to genetic factors or to the experimental conditions was t determined.

Above 25 μ in length the oscular and basal rays grow at similar rates, regardless of hether the rays belong to Y-, T-, or even aberrant spicules. The orientation of the tic axis of the mineral constituent has thus no appreciable effect on the growth rate.

INTRODUCTION

PRECISE figures for the rate of growth of calcareous sponge spicules have not been published. Maas (1900) deduced that monaxons grow rapidly ecause young specimens of *Sycandra setosa* suddenly develop a prickly pearance, but he apparently overlooked the possibility of a rapid pro-usion of fully grown spicules through the surface. Hammer (1908) also erely states that monaxons of *Sycon raphanus* grow rapidly in length withut giving precise data. Triradiates were believed to grow more slowly (Maas, 900) and some idea of their growth rate is afforded by their appearance in the rvae of *L. variabilis* (Minchin, 1896) and *Clathrina blanca* (Minchin, 1898) n the second day after settlement. More recently Sasaki (1941) has calulated the relative growth rates of spicule rays from measurements made on mples taken from specimens of *Sycon okadi* of various sizes. Again absolute tes are not given, but if the rays continue to grow throughout the growing eriod of the sponge, as Sasaki assumes, a slow rate would be implied. This ssumption is, however, questionable, because it is obvious from some of the rawings (for example, his fig. 10) that the larger spicules depicted could not ave grown from the smaller ones in the same series, since considerable hange in shape would be involved besides a mere increase in ray length.

The absence of small spicules in the samples from the larger sponge is ossibly due to their relative scarcity in proportion to the total number of picules present. Furthermore, some of the small spicules may have been lost, ince those which were formed when the sponge was small would not fit the

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curvature of the wall in the larger specimens. Small monaxons in particul could easily be shed. The elimination of badly fitting spicules through t surface has been noted in species of *Leucosolenia* (Jones, 1954*b*), and the poss bility of spiculoclastic activity must not be overlooked: Prenant (1925) h found detached porocytes with the remains of spicules included in their cyt plasm in the gastric cavity of *Clathrina coriacea*.

The most satisfactory way of determining the rate of spicule growth is record the growth directly by photographic means. This has now been don with pieces of the wall of *L. variabilis* (Minchin, 1904). This species is ideal f the purpose, since its oscular tubes are comparatively free from diverticu and the wall is transparent. As described below, the pieces were stretche between clips under a $\frac{2}{3}$ in. objective, the photographs being taken by mean of conventional photomicrographic apparatus.

Apart from spicule growth, the photographic records provide usef information concerning the properties of the various components of the wal These will be described in a later paper.

METHOD

The apparatus is depicted in fig. 1. A piece of the wall of *L. variabilis* i held between a pair of clips which rest on a glass plate lying on the bottom o a pyrex dish of diameter 10 cm and sides 5 cm high. The dish rests on microscope stage beneath a $\frac{2}{3}$-in. objective, which is protected from the sea water in the dish by a glass cap held on by plasticine (fig. 1, A). The ca consists of a coverslip sealed on to a glass sleeve by means of araldite, th coverslip being set about 3 mm below the surface of the objective lens. Th use of the coverslip avoids an air-water interface which might ripple or collec dust. During the experiment (except when photographs were being taken the sea-water in the dish is circulated by a stream of air bubbles introduce through a glass tube clipped to the side (fig. 1, C).

The piece is stretched by brass washers hanging outside the dish, which ar attached to the clips by means of cotton thread and glass hooks. The threa is bent round glass rods, 1 mm thick, which are fused together to form a rigi framework resting on the rim of the dish. The weights used (including th hooks) vary up to 0·7 g on each side.

For the manufacture of the clips (fig. 1, B) solid soda-glass rods of 9 m diameter were drawn out into thin lengths of 1 mm or less in thickness. Th lengths were bent into the required shape by means of a 1 mm flame, th pieces 1 mm thick being used for the spring framework of the clip and thos 0·3 mm thick for the jaws. The jaws were set so that they were sprun together, but could be separated by light pressure on the top of the clip. Th width of the jaws was about 1 cm and the total length of the clip 2·7 cm.

The method of mounting the piece was as follows. An expanded oscula tube of *L. variabilis* was excised and cut along one side. With one corne gripped by forceps, one of the cut edges was inserted between the opene jaws of a clip resting on the platform, and the jaws were allowed to com gether when the piece was correctly sited. The piece was then uncurled th the aid of a fine glass needle and the other cut edge slipped between the ened jaws of the other clip, this being pushed nearer its partner in the ocess. The threads were then hooked to the clips and weights were added. t the end of the experiment the piece was released, flattened against a glass

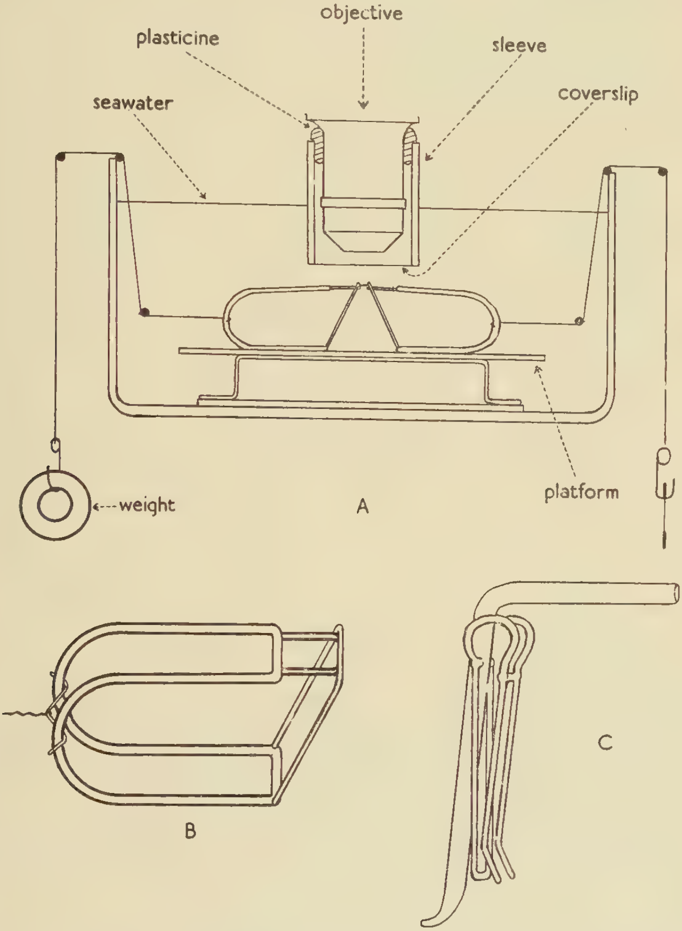

FIG. 1. A, diagram of the apparatus in use. B, a clip drawn in perspective. C, aerator-tube which was fixed to the side of the dish by means of the glass clip.

ide by lifting this out of the water, and fixed with a few drops of 90% cohol. The tissue tends to sever between the jaws on each side and this evented the pieces from being held in position for more than about 17 h.

Overlapping areas of the piece were photographed at intervals of roughly h by means of a Leitz–Wetzlar 'Makam' camera. Ilford HP3 plates and 2 developer were used. The top lens of the condenser on the microscope as removed. Illumination was by a 200-watt Mazda photoflood bulb, set about 6 in. from the microscope mirror. A series of filters was interposed the beam as follows; (*a*) Chance ON20 (heat absorbing), (*b*) Ilford 'Q' (u light absorbing), (*c*) Ilford B401 (transmitting green). While the microscop was being focused an additional blue glass filter was inserted and the illumin tion was considerably reduced by switching in an additional 200-watt bulb

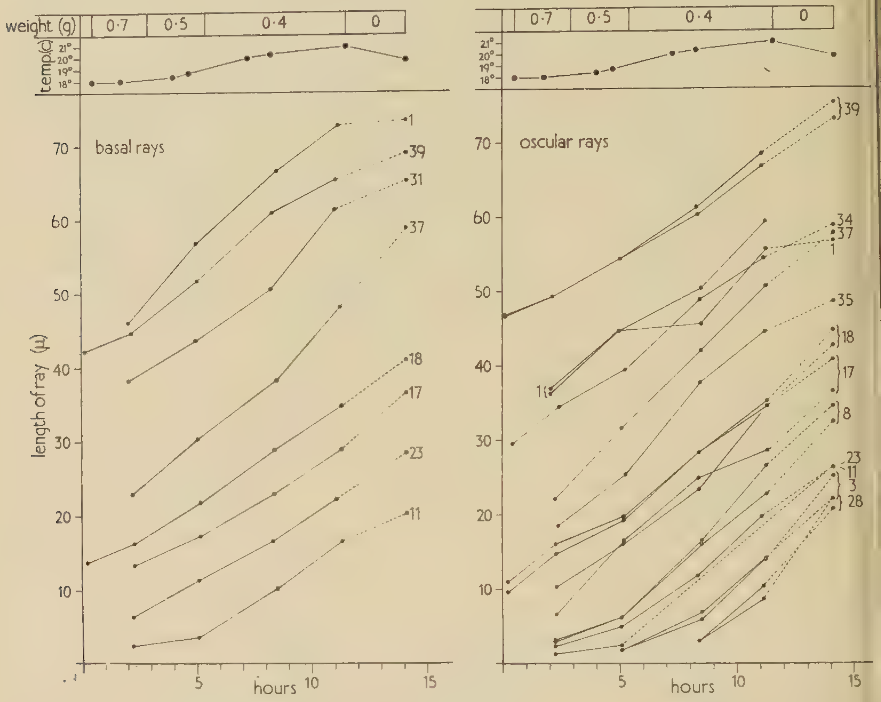

FIG. 2. Typical growth curves for basal rays (left) and oscular rays (right) of growing spicule in piece A. The spicules were numbered and their numbers are given on the right-hand sid of each graph. The measurements at 14 h were made directly on the fixed piece. All othe were made on enlarged photographic prints. Some spicules, e.g. spicule 1, apparently cease growing during the last period (11–14 h). Note that the growth is slower for rays of less tha about 6 μ in length, but the rate rises to a fairly uniform level subsequently. Note also th the oscular rays tend to be smaller than the associated basal ray when small, but become rel tively larger, or similar in size, as they grow. Photographic records of the growth of spicul 31 and 39 are given in fig. 3.

At the top of each graph is a record of the weight on each side of the dish which was used stretch the piece. A record of the temperature of the sea-water is also provided.

series with the lamp. Exposure times less than a second, obtained by flickin the switch on and off, were employed.

Enlarged prints were used for measurements of the lengths of the rays growing spicules. On the enlarged print 1 cm corresponded to a distance 25 μ. The measurements were accurate to within 0·5 mm, though erro sometimes arose from the difficulty of determining correctly the position the ray tip, either because it lay beneath another ray, or because visible trac of the secreting cell at the tip confused the picture. However, most of th

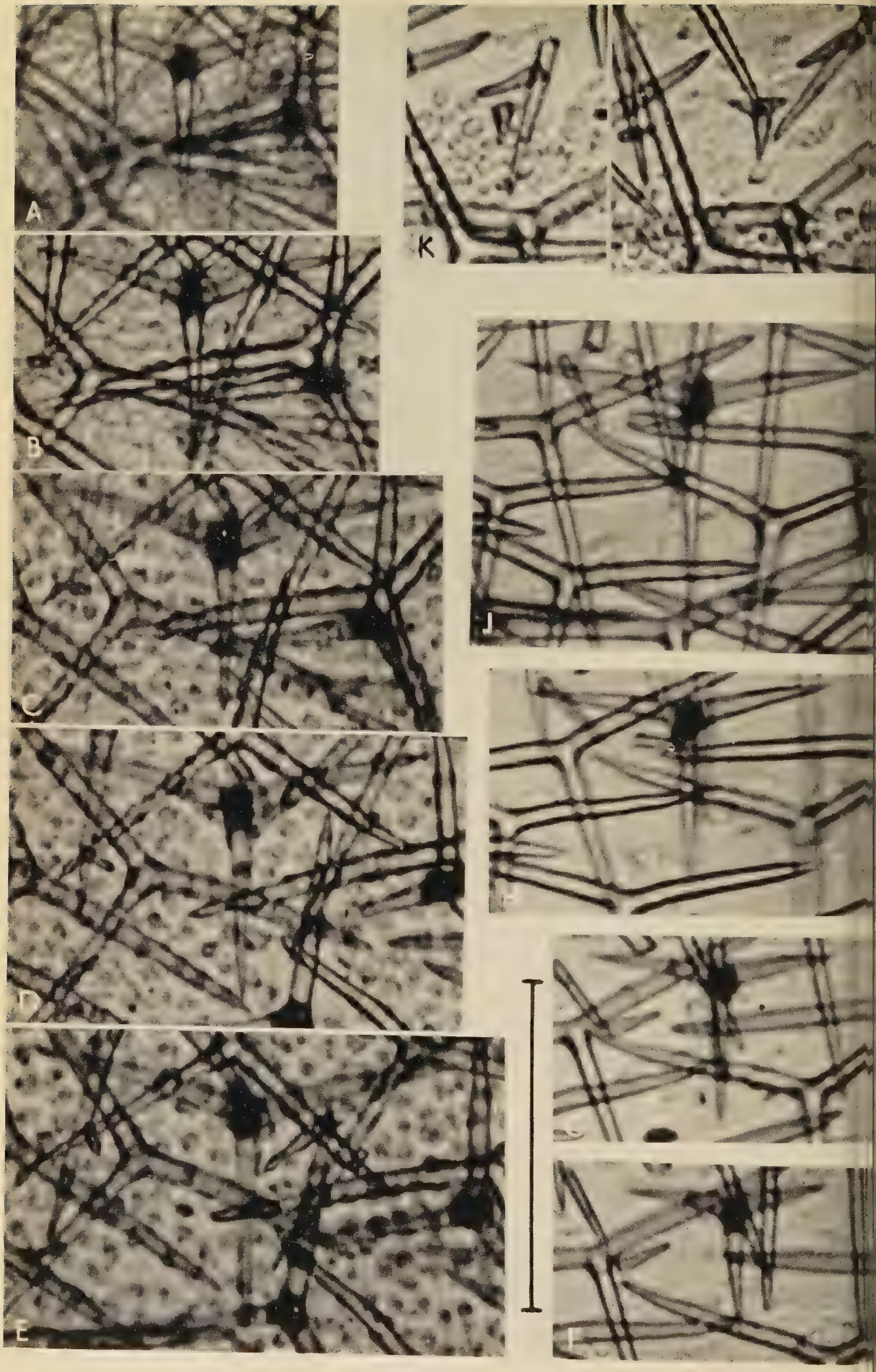

FIG. 3

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cules gave consistent results and errors due to rays being slightly tilted, slightly out of focus, were hardly appreciable (usually much less than 2%) en determined by comparing the photographs with the same spicules in fixed preparation. The rays were all measured from the central point of ersection of their axes.

RESULTS

Good crops of growing spicules were obtained on three pieces of the wall. ese will be designated A, B, and C respectively. A supplied the best crop d most of the following account deals with this. The piece was mounted in e August 1952, one year after the other two pieces. B and C are of addi- nal interest, however, since they were both derived from the same oscular e, C being mounted about one week later than B.

Spicule growth in piece A. Piece A was initially 2·79 mm long by 0·44 mm de and was stretched 4 to 5 times in width by the weights before these were noved completely, 11½ h after the start. The piece was photographed just er mounting, again after a period of 2 h, and then at 3-hourly intervals. It e across some time between 11½ and 14 h after the start and then contracted d curled somewhat before it was finally fixed at about 14 h. The tempera- e of the sea-water ranged from 18° to 21° C.

In fig. 2 the lengths of the rays of growing tri- and quadriradiates are tted against time. To avoid confusion not all the spicules measured have en plotted. The measurements at the time of fixation (14 h) were made ectly on the spicules in the piece by means of a calibrated eyepiece micro- ter. During the last interval some of the rays stopped growing, probably a result of the mechanical upset consequent upon the contraction and rling of the piece. Stages in the growth of selected spicules are shown in s. 3 and 4.

The curves in fig. 2 indicate that the growth rate of both oscular and basal s is low at first and then rises to a steady level. This would be the case if growth followed the typical S-shaped curve, but whether there is in fact orresponding decline in growth rate as the rays approach their maximum gth is not certain. The act of fixing the pieces in position stopped growth the larger spicules present, and it was not possible to follow the growth of one ray through to the fully grown condition since the pieces could not be intained in position for more than 17 h.

The growth rates of all the growing spicule rays were determined for each erval from the change in length of the ray and the duration. The maximum es calculated were 5·26 μ per h for basal rays and 6·22 μ per h for oscular s, but these figures are probably the result of a fortuitous combination errors and are almost certainly too high. The average rates for different

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IG. 3 (plate). Photographs of growing spicules. The line indicates 100 μ. A–E, a Y-shaped driradiate (spicule 39).
–J, a T-shaped quadriradiate (spicule 31).
–L, early stages in growth. The basal ray at first grows precociously.

TABLE 1

Growth rates of basal and oscular rays of symmetrical tri- and quadriradiate piece A

The upper figure in each space is the average growth rate in μ per h for the period. The figure in brackets beneath is the number of measurements from which the average was calculated. Beneath this figure is the standard deviation. The data for period V were not included when calculating the overall averages because the piece contracted and curled during this period and many spicules stopped growing

Mean length	Period:	*I*	*II*	*III*	*IV*	*V*	*Over*
	Hours:	0–2	2–5	5–8½	8½–11¼	11¼–14	*avera*
	Temp., °C:	18	18–18·9	18·9–20·4	20·4–21	21–19·8	*I–I*
1·5–5 μ	Basal rays		0·34 (1) 0				0·3 (1 0
	Oscular rays		0·86 (6) 0·31	1·33 (2) 0·14			0·9 (8) 0·3
5+–25 μ	Basal rays	1·19 (1) 0	1·21 (5) 0·6	1·98 (6) 0·24	1·94 (7) 0·41	1·45 (6) 0·85	1·7 (19 0·5
	Oscular rays	2·26 (2) 0·05	1·98 (8) 0·78	2·36 (8) 0·75	2·94 (11) 1·05	2·67 (10) 1·02	2·4 (29 0·9
>25 μ	Basal rays	1·23 (2) 0	2·45 (8) 0·96	2·23 (10) 0·52	2·88 (10) 1·29	1·78 (10) 1·00	2·4 (30 1·0
	Oscular rays	1·57 (4) 0·52	2·43 (13) 0·82	2·33 (17) 0·86	2·92 (21) 1·08	2·18 (20) 1·10	2·5 (55 1·0

categories of ray are given in table 1. The rates calculated for the last inter are unsatisfactory since some of the rays gave low growth rates probab owing to cessation of growth at some time during this period.

In fig. 5 the growth rates for oscular and basal rays are plotted against t mean length of the rays during one of the intervals (Period II). All the ra concerned would have experienced similar conditions of temperature, lig &c., so that differences in growth rate may be directly correlated with t

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FIG. 4 (plate). Photographs of growing spicules. The line indicates 100 μ.

A–B, triradiate.

C–D, early stages in growth of asymmetrical triradiate.

E–J, triradiate. Note precocious growth of the basal ray and subsequent equalization of rays.

K–L, an aberrant spicule caused probably by rotation of the primordium about the axis the basal ray.

M–O, aberrant spicule also caused by abnormal setting of the cell-sextet or primordium

P–R, aberrant spicule with unequal oscular rays.

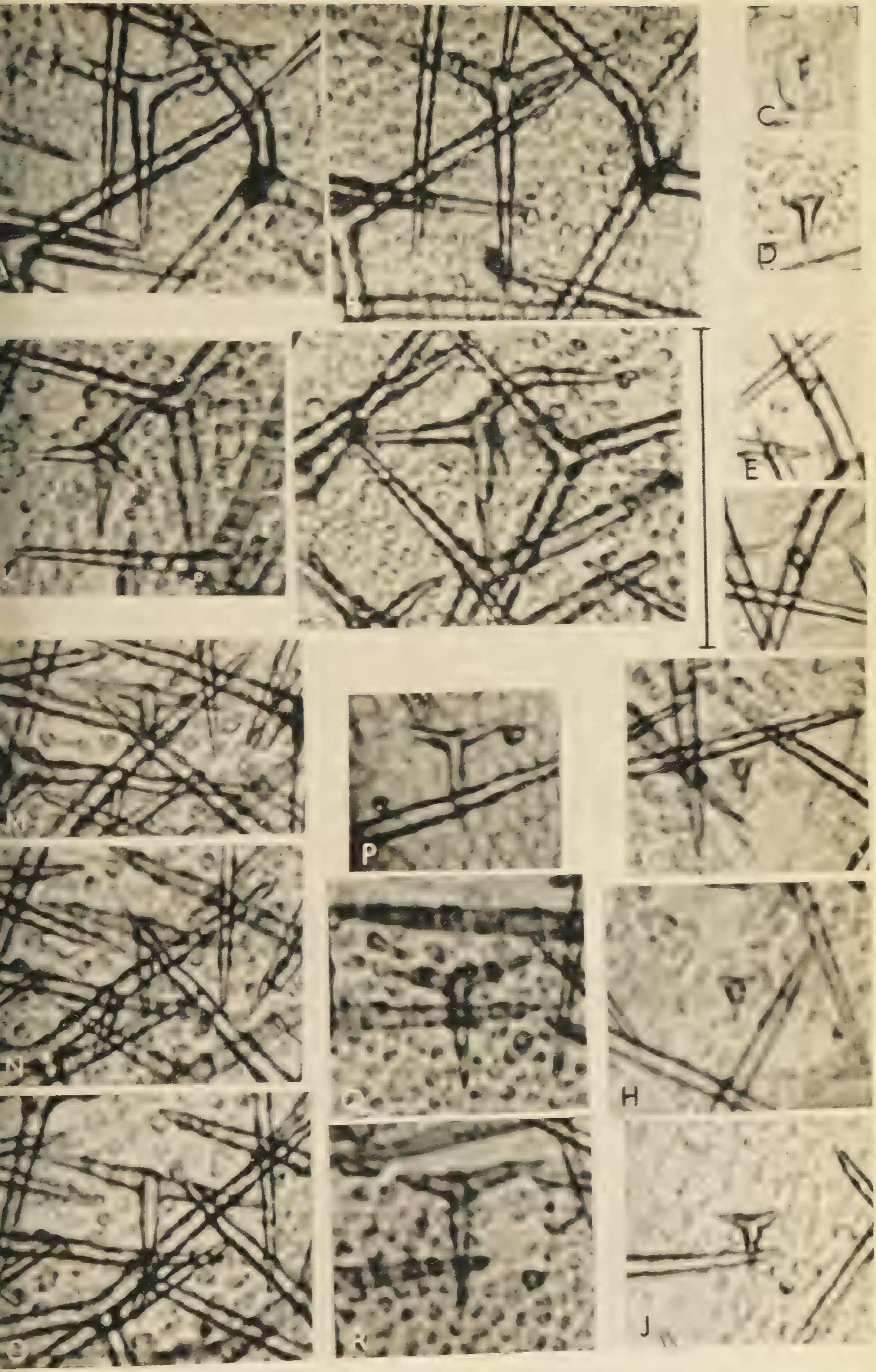

FIG. 4

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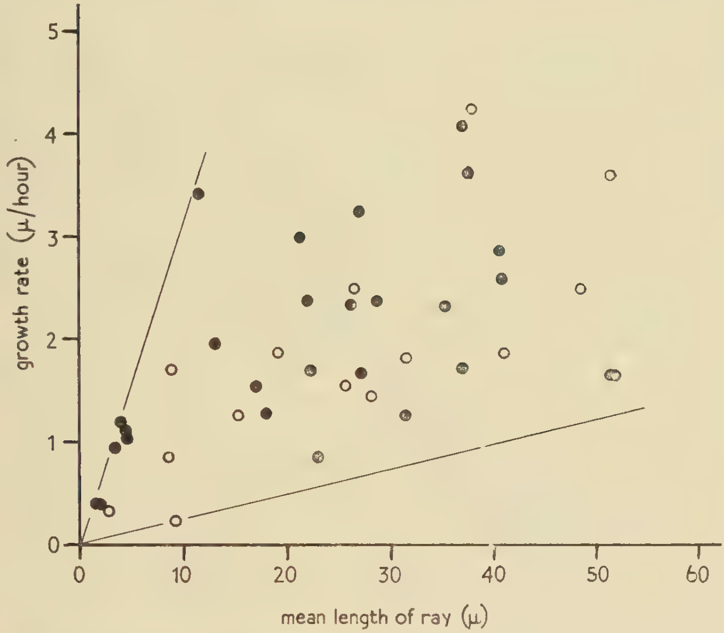
ean length of the rays. The graph confirms what was noticed in fig. 2, mely, that there is initially an increase in rate as the rays increase in length.

Fig. 6 is a graph showing the relation between the length of the basal ray d the length of each associated oscular ray at various stages of growth. nce the points for the rays above 20 μ long are evenly scattered about a line clined at 45° to the abscissa, it is clear that the growth rates of oscular and



IG. 5. Graph showing the relation between the growth rate and the mean length of the rays ring Period II in the photographic record for piece A. The rate increases with the growth of the ray. ○, basal rays; ●, oscular rays.

asal rays above 20 μ in length must be almost identical. Below 20 μ the basal ys first grew precociously and were later caught up by the oscular rays. The ecocious growth of the basal ray is obvious from the shape of the primordium (figs. 3, K; 4, C, G), and was noticed by Minchin (1908) for spicules L. *complicata*. For *L. variabilis*, however, he states that there is 'no such ecocious development of any one of the three rays'.

Table 1 gives the average growth rates for rays grouped according to length to three categories, 1·5 to 5 μ, 5+ to 25 μ, and > 25 μ. The rate for the sal rays in the range 1·5 to 5 μ is lower than for the corresponding oscular ys, and the same is found with the average rates for the range 5 to 25 μ: for e basal rays the overall average is 1·72 μ per h, whereas for the oscular rays is 2·47 μ per h. These results are significantly different ($P > 0{\cdot}1 < 0{\cdot}5\%$). r rays with mean length greater than 25 μ, the overall rates were respecely 2·44 and 2·53 μ per h. These are not significantly different ($P > 50\%$). us after the initial period when their growth was severely impeded, the oscular rays rapidly reached their maximum rate of growth and continued this rate whilst the basal rays were taking longer to reach their maximum ra Both types of ray had then roughly the same average maximum rate. Th there is agreement with Minchin's deduction (based on a comparison

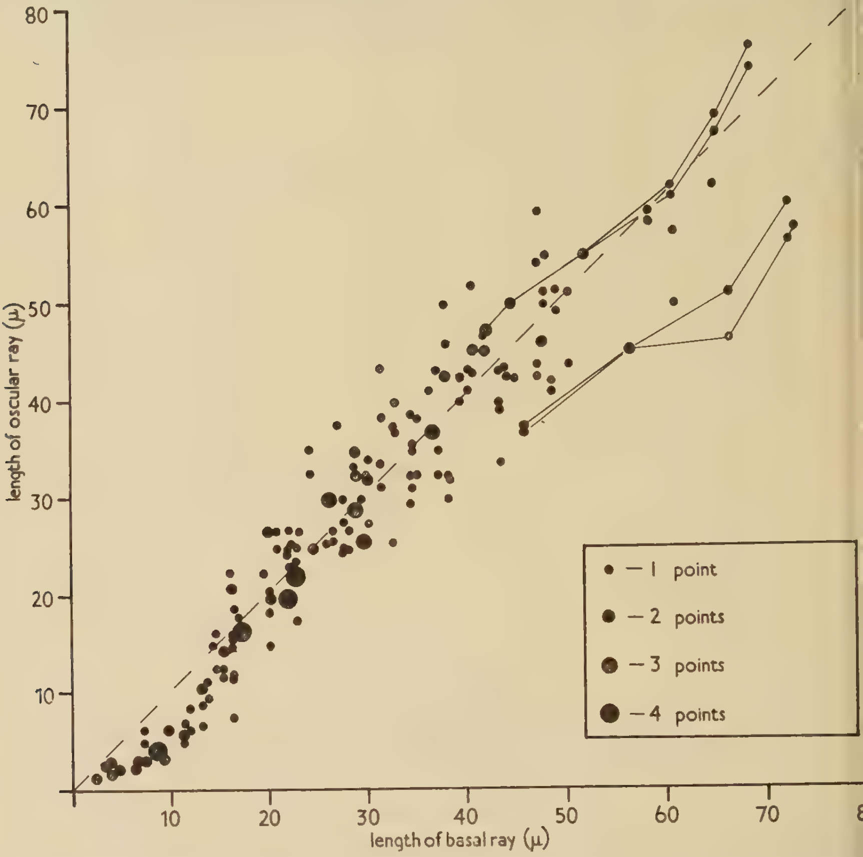


FIG. 6. Graph showing the relation between the length of the oscular rays and the lengt the associated basal ray of growing spicules in piece A. The oscular rays are shorter than basal ray at first, but they soon catch up and then both types of ray maintain similar si The points which are interconnected by lines relate to two particular spicules (1, below; above). Note that above a length of about 20 μ the oscular and basal rays tend to be simila length, being uniformly scattered about the 45° line, and even when dissimilar, as with spic 1, the growth rates of the two types of ray must be much the same.

growth stages) that the oscular rays of *L. complicata* rapidly reduce the proportion between them and the basal rays.

Many of the growing spicules were aberrant in form, the oscular rays be unequal in length, or one being kinked, or the rays being set at abnor angles to each other (fig. 4). The growth rates of the unkinked rays of more obviously asymmetrical aberrants are given in table 2. In no case is th a significant difference at the 5% level between these rates and those for ys of similar length and period in Table 1. Furthermore, the oscular rays T-spicules grow at similar rates to those of Y-spicules (for example, icules 31 and 39 in fig. 3).

In table 1 the average growth rates for the first interval tend to be lower an those for the other intervals, though statistically the differences are not l significant at the 5% level. Only with the basal rays of the 5 to 25 μ range, d the oscular rays of > 25 μ length, is there a significant difference between e average rate for the first interval and the highest average in all the subse- uent intervals. The apparent increase in growth rate may have been caused y the reduction at 3 h in the weights producing extension, or alternatively, e calcoblasts may have become adapted to the new conditions of growth fter initial inhibition due to the application of tension to the wall. The rise in mperature is unlikely to account for the whole of the increase. Assuming a Q_{10} of 2·5, a rate of 1·19 μ per h would be expected to increase to 1·30 μ per h r a 1° C rise in temperature, and a rate of 1·57 to 1·72 μ per h; both expecta- ons fall short of the largest average rates actually measured.

TABLE 2

rowth rates of oscular and basal rays of aberrant spicules in piece A (kinked rays excluded)

The top figure in each space is the average growth rate in μ per h for the period. Beneath in brackets is the number of observations from which the average was calculated. Beneath this is the standard deviation

Mean length	Period:	*I*	*II*	*III*	*IV*	*V*
–25 μ	Basal rays	1·97 (3) 1·28	2·16 (3) 0·44	1·80 (1) 0	2·47 (1) 0	0·60 (1) 0
	Oscular rays	2·51 (3) 1·44	3·32 (2) 0·02	2·56 (2) 0·11	2·11 (1) 0	2·12 (1) 0
25 μ	Basal rays		2·75 (2) 0·025	1·38 (2) 0·54	3·25 (3) 0·71	1·05 (4) 0·55
	Oscular rays		2·42 (2) 0·13	2·28 (4) 0·50	2·86 (6) 0·80	1·74 (8) 0·82

Spicule growth in pieces B and C. Piece B was initially about 2·1 mm long y 0·5 mm wide and was mounted for 17 h. The piece was not clipped satis- ctorily, the wall being slightly puckered, so that some regions became more retched than others. However, the weights were soon reduced and then emoved completely at about 6½ h from the start, so that the width only ncreased to 3 times the initial size. Even after the weights had been removed e stretching continued for half of the total increase, but this can be explained by assuming that the clips had been lifted at their ends by the pull of weights, and had then settled under gravity after the weights had b removed, thereby maintaining tension on the piece.

Piece C was derived from the distal end of the same oscular tube as though one week later. It was a narrow piece (2·16 mm long by 0·26 mm wid which became greatly stretched while held by the clips (about 17 h). T width in fact increased 7½ times. The piece had a small cut at one end a part of the wall under one clip slipped free, so that a considerable change shape took place. The weights were reduced and then removed at 11¼ h. T temperature of the sea-water was 17·1±0·5° C, whereas for piece B it v 18·1±0·7° C.

TABLE 3

Growth rates for oscular and basal rays of tri- and quadriradiates in piece (above) and piece C (below)

The top figure in each space is the average growth rate in μ per h. In brackets beneath is the number of observations used for calculating the average. Beneath this is the standard deviation. Altogether there were 3 intervals between the photographic series for piece B and 5 for piece C, but there were no significant differences between the average rates for each period in either case. Thus all the calculated growth rates have been lumped together regardless of period for the determination of the averages given in the table.

	Length:	1·5–5 μ	5+–25 μ	> 25 μ	*Overall average* > 5 μ
Piece B	Basal rays		1·6 (8) 0·58	1·6 (14) 0·60	1·60 (60) 0·71
	Oscular rays	0·12 (1) 0	1·53 (17) 0·59	1·66 (21) 0·89	
Piece C	Basal rays		1·39 (9) 0·49	1·36 (20) 0·69	1·21 (63) 0·61
	Oscular rays	1·57 (1) 0	0·97 (19) 0·47	1·21 (15) 0·61	

For both pieces there were no significant differences (at the 5% lev between the average growth rates of either basal or oscular rays for each s cessive interval throughout the experiment. All the interval rates have the fore been grouped together and used for the calculation of the avera displayed in table 3. In this the differences between the oscular and basal r of the same size-range are not significant ($P > 50\%$), except for the rays 5 25 μ long in piece C, in which the difference is just significant at the 5% lev he oscular rays 5 to 25 μ long in piece C grew more slowly than the correponding basal rays.

When the lengths of the oscular rays of growing spicules were plotted ainst the length of the basal ray of the same spicule, practically all the points y to the right of the 45° line for both pieces; the oscular rays tended to be naller than the associated basal ray. This result may be compared with the aph in fig. 6 (piece A). Here the rays tend to be similar in size when only μ or more in length. The difference may be due to the conditions of the periment, because the oscular rays of fully grown spicules in both B and C ere often larger or as large as the basal ray on the same spicule.

If the average growth rates of either the basal or oscular rays of piece B are mpared with the corresponding rates for C, a significant difference is only be found between the respective oscular rays of mean length 5 to 25 μ $> 0{\cdot}1 < 0{\cdot}5\%$). The low average growth rate in C may have been due to e severe stretching suffered by this piece.

When the growth rates for all the oscular and basal rays of mean length eater than 5 μ are averaged, a significant difference between the two pieces 3 and C) is found ($P > 0{\cdot}1 < 0{\cdot}5\%$). The rays in C grew more slowly than ose in B on average, even though both pieces were derived from the same cular tube. The 1° C lower temperature in the case of the former would oubtless contribute, though it probably would not account for the whole of e difference; assuming a Q_{10} of 2·5, the average growth rate would be xpected to increase from 1·21 μ per h to 1·33 μ per h, whereas in B the erage rate was 1·60 μ per h. Again, the severe stretching in piece C may ave been responsible for the reduction in growth rate of the rays.

When compared with the data for piece A, the average rates for the rays B and C are low. Again the difference can be partly attributed to temperaure, for the range with A was 18° to 21° C. Otherwise the difference in verage growth rates may have been due to differences in genetic constitution, possibly to differences in relative age of the material. Piece A was mounted n 22 August 1952, whereas pieces B and C were mounted on 27 August 1951 nd 3 September 1951 respectively. The growth rates of the spicules may fall ff as the material ages and the season draws to its close.

As with piece A, the maximum growth rate for a given length of oscular or asal ray in B and C tended to increase as the mean length increased, but the ata are far too few to warrant this observation from the results of B and C alone.

Discussion

The results obtained with the three pieces are not fully consistent. In all hree the basal ray grew precociously at first, and the growth rate of both ypes of ray tended to increase to a steady level as the spicule rays lengthened, ut the average growth rates were quite different. For rays of more than 25 μ length the average rates were 2·50 (A), 1·64 (B), and 1·29 (C) μ per hour. he differences were greater than would be expected from the difference in mperature.

Another inconsistency is that in A the oscular rays in the range 5 to 25 grew faster than the corresponding basal rays, whereas in C the reverse w just significantly the case, and in B there was no significant difference b tween the two types of ray. The 3 rays of the spicules in A also tended to b about the same in length when longer than about 20 μ, whereas in both B an C the oscular rays of the growing spicules (but not of the fully grown spicule tended to be smaller than the associated basal ray.

These differences may be due in part to differences in genetic constitutio between A on the one hand, and B and C on the other. This is not necessaril so, for B and C were derived from the same oscular tube and yet provide dissimilar data. Further experiments are needed to elucidate the effects o stretching, temperature, and age on the mounted pieces.

The growth in all three pieces of asymmetrical spicules with one oscula ray longer than the other, or with a kinked oscular ray, is one effect of th stretching process. Their aberrant form is the result of an abnormal arrangement of either the formative cell-sextet or the spicule primordium, as woul result from a shear between the mesogloea and internal epithelium of the wa (Jones, 1954*a*). Such shearing would be caused by local differences in extensibility of the stretching materials. The inhibitory effect on spicule growth o the contraction and curling of piece A (which could also result in shearing has already been noted, as has also the increase in growth rate following th reduction in the tension applied to this piece. As the calcoblasts are in contac with the inner epithelium, the transverse stretching and associated longitudinal shortening of the epithelium would quite possibly influence the rate of growth of the different rays.

Initial differences between the growth rates of the basal and oscular ray are possibly due to the tilting of the formative cell-sextet (Jones, 1954*a*, 1958 This would direct the oscular ray primordia against the inner epithelium an also perhaps squash the oscular ray founder-cells beneath their associate thickener cells. As a result the growth of the oscular rays might well b inhibited at first. Later, when the founder-cells had progressed beyond th border of the overlying thickener-cells and the growth of the oscular rays ha become parallel to the choanoderm, growth might be speeded up considerably. The initial tendency for growth towards the choanoderm might als result in a lifting of the central junction of the three rays, which in turn woul involve a change in the direction of growth of the basal ray, so that this to would suffer some inhibition. The growth of all three rays would thus b subject to interacting restraints which might well account for the differenc between the respective rates for the basal and oscular rays. Evidence for lifting of the central junction during the secretion of the proximal 30 μ of th rays of triradiates (and some quadriradiates) has already been provided (Jone 1954*b*).

If the initial rudiments of the oscular rays are tilted against the choanoder the question arises whether there is any appreciable error in the calculate growth rates as a result. If θ is the angle the ray makes with the surface, th

tual length corresponding to the measured length L would be $L/\cos\theta$, and
e true rate of growth would be $1/\cos\theta$ times the measured rate. For the
cular rays the angle θ is given by the equation $\sin\theta = \cos\alpha\cos\beta$, where α
½ the planar angle and β is the optic angle (Jones, 1954*b*). For quadriradiates
L. complicata typical values for the planar angle and optic angle are respec-
ely 150° and 25° (Jones, 1954*b*). If one assumes extreme values for these
gles in spicules of *L. variabilis*, namely, 120° and 20°, the factor $1/\cos\theta$
comes only 1·132, so that the actual growth rate of the oscular rays would
only 1·132 times the measured growth rate. Furthermore, this error would
ly be possible with rays of less than 9½ μ in length, since the central
nction of the spicule could not be higher than about 5 μ from the choano-
rm ($5/\tan\theta = 9{\cdot}5$); 5 μ is about the thickness of the calcoblasts and about
lf the thickness of the fully formed spicule rays. For rays longer than 9½ μ
e factor $1/\cos\theta$ would approach unity and the error would diminish. If
ore probable figures for the optic and planar angles are taken, namely, 25°
d 150° respectively, the correction factor $1/\cos\theta$ becomes 1·028 and the
ror is less than 3% for rays of less than 20·8 μ in length. Thus for most of
e data the possibility of error due to the direction of growth not being in the
cal plane of the camera can be neglected. The difference between the rates
r oscular and basal rays 5 to 25 μ long in piece A was about 30% of the rate
r the former. For the basal rays, the growth is directed along the surface
om the first and the angle θ would be very small.

It is interesting that the oscular and basal rays should grow at the same
tes when more than 25 μ long. The optic axis of the spicular substance is
ughly transverse to the direction of growth in the former, whereas in the
tter it is nearly in the same direction, but inclined at an angle of about 20–
0° (Jones, 1955). This suggests that the growth of the rays is limited by
ctors other than the rate of crystal growth, since different crystal faces would
resumably be growing at the tip of each type of ray and it is likely that the
rystallizing solution is the same in all cases. The similarity between the
rowth rates of rays of aberrant spicules and those of normal spicules leads
o the same suggestion, since in the former the relation between the direction
f growth and the optic axis would be very variable. Whether in fact the
ifferences in growth rate between the respective crystal faces would be de-
ectable, however, would depend upon the composition of the crystallizing
olution, for example. At all events, the results do not necessitate any modifica-
ion to the explanation of spicule form given in a previous paper (Jones, 1955).
ncidentally, the growth rate (2·5 μ per h) is much less than the recorded
peeds of amoeboid movement (0·5–4·5 μ per sec) (Prossor, 1950, p. 633),
hough if such movement were involved it might be hindered by the meso-
loea. However, there is evidence that the spicules grow where the meso-
loeal substance is relatively fluid (Jones, 1956).

Assuming that the growth rates of the spicules in the normal conditions of
fe are similar to those measured in the stretched pieces, it can be calculated
hat it would take between one and two days for a quadriradiate to grow to

maximum size. Such has been found to be the case by photographica recording the growth of whole oscular tubes. An account of this work w form the basis of a subsequent paper.

Once again it is a pleasure to record my indebtedness to the Director a Staff of the Marine Biological Laboratory, Plymouth. The photograph records were obtained there, whilst I was allowed the facilities of the Cambridge University Table, for which I am also grateful to Professor Sir Jam Gray.

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A Note on Berenbaum's Acetone / Sudan Black Technique for Bound Lipids applied to Insect Cuticle

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SUMMARY

Colouring by Sudan black in acetone at 37° to 60° C is not a histochemical test for bound lipids.

IN the course of a study upon the secretion of wax through the cuticle of the larva of *Calpodes ethlius* Stoll (Lepidoptera, Hesperidae), the possibility was considered that the wax might be transported while attached to a protein as 'bound lipid'. Berenbaum (1958) gives three methods for using Sudan black at high temperatures (37° to 60° C) in acetone or 70% ethanol, which he supposes to demonstrate 'bound lipids' not readily shown by other means. His acetone / Sudan black technique was therefore applied to formalin-fixed sections and blood-smears of the caterpillar. The endocuticle coloured strongly, but not the exocuticle and epicuticle. The blood-smear was also coloured at the time when it contained the carbohydrate and protein precursors of the endocuticle. The extensive literature on cuticle structure describes lipids in the outer layers but none in the endocuticle (Richards, 1951; Wigglesworth, 1939 &c.). This surprising result prompted a brief further investigation of the validity of the technique.

Berenbaum gives as one proof of the specificity of his method the failure of tissues to colour after extraction for 70 days in methanol / chloroform. Cuticle sections were refluxed for 77 days in 1 : 1 menthanol : chloroform in a Soxhlet extractor. There was no reduction in the intensity of the colouring of the endocuticle or any other tissue in the section—oenocytes, fat-body, muscle. This strongly suggested that the Sudan black was demonstrating something other than 'bound lipid'.

An essential preliminary to successful colouring by the acetone / Sudan black method is the 'unmasking' of the 'bound lipids'. Berenbaum suggested washing in tap-water for 2 to 24 h as the most easily controlled way of doing this. Now one of the effects of prolonged washing would be the hydration of the tissue proteins. Subsequent dehydration in alcohol might replace the water-filled spaces, so that the tissue would have a porous structure. The colouring of such a tissue by Sudan black might then be a process of infiltration of the spaces. This hypothesis would account for the observation that there is a slow loss of colour in organic solvents. If the 'unmasking of bound lipids' is in reality a process of hydration, changing the physical structure to allow the penetration and retention of the dye, then it should be possible to

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reverse the effect by drying out in air rather than dehydrating in alcohol. Th hypothesis was tested on cuticle sections and blood-smears. The 'unmasking effect of washing was completely reversed by drying out for 30 min in a 50° C oven, or in a vacuum desiccator at room temperature, or even by leaving th slide on the bench. The 'unmasking' effect was not reversed by dehydratio in absolute ethanol or acetone even for several hours at 50° C. This might b interpreted to mean that the physical process of drying out also 'remasks' th bound lipids. Until the chemical or physico-chemical basis of 'unmasking has been elucidated, the hydration hypothesis would seem to be the simple one, for 'unmasked lipids' appear only to be recognizable by sudanophilia.

If the colouring of the endocuticle is due to its physical structure, it shoul be possible to create a lipid-free model with the same colouring reactions. A sample of pure chitosan was prepared from the cuticle by digesting in saturate aqueous potassium hydroxide at 160° C for 30 min. The chitosan was washe for several hours in running water. After dehydrating in alcohol the chitosa was coloured strongly by Sudan black. Another piece of chitosan dehydrate by drying in air failed to colour. Thus the physical properties are all-importan and a tissue need not contain protein or lipid to be coloured.

In the insect tissues examined, the acetone / Sudan black technique doe not reliably indicate bound lipid since:

(*a*) it does not colour the outer parts of the cuticle where bound lipids ar known to occur;
(*b*) it may colour the endocuticle where there is no other evidence for th presence of lipid, but it only does so if the tissue has been previousl hydrated;
(*c*) the treatments described as 'unmasking bound lipids' would be expecte to induce the hydration of proteins and polysaccharides;
(*d*) chitosan containing no lipid colours strongly, provided that it has bee previously hydrated;
(*e*) the colouring is not reduced by prolonged extraction in organic solvents.

The demonstration that the acetone / Sudan black method colours a hydrate polysaccharide and presumably also hydrated tissue proteins, does no preclude the possibility that it may also colour lipids bound to proteins, bu the unmasking agents in use, for example, pepsin, trypsin, soaps (Berenbaum 1958), dilute ethanol, salts of mercury, cadmium, zinc (Ciaccio, 1926), phen (Gupta, 1958), might all be expected to hydrate the tissues as fast as or faste than water alone. Some of them might also create a porous colourable structur by the removal of lipids or some other tissue component. Colouring by Suda black in acetone after their use, while useful and repeatable histologically has a dubious histochemical significance.

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An Account of Larval Budding in the Compound Ascidian, *Hypsistozoa fasmeriana*

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With three plates (figs. 4 to 6)

SUMMARY

Larval budding in *Hypsistozoa fasmeriana* is in many ways unique in the sub-family Holozoinae. The stolon, which projects from the left side of the oozooid, is large (235 μ in diameter, 1·8 mm in length) and reaches maximal size before severance of buds occurs. The buds arise one at a time and 9 to 14 are formed.

Test forms rapidly between a newly severed bud and the remainder of the stolon. Thus the buds are moved along an arc of a spiral which runs from the left side of the oozooid somewhat anteriorly across the ventral side and posteriorly up the right side. By the end of bud formation the first-formed bud occupies the most posterior position, lying high up on the right side of the oozooid.

Each larval bud develops directly into a blastozooid and by the time the tadpole becomes free-swimming there is a considerable degree of organogenesis. The blastozooids together with the oozooid form a ring of zooids tilted slightly away from the vertical. After metamorphosis of the tadpole this ring becomes horizontal, but the tilt is still maintained with the oozooid occupying the most elevated position. Thus in the young colonies the plane of the head is slightly off the horizontal—an arrangement which persists throughout the life of the colony.

The development of larval buds in this ascidian is not delayed until after de-differentiation of the oozooid, as is the case in the other Holozoinae. The blastozooids function simultaneously with the oozooid. They do not, however, become sexually mature, being presumably of sub-maximal size for the species.

The newly severed bud differs from that of other Holozoinae in having an extensive epicardial tube and a thick mesenchymal layer of densely granulated cells. The epicardium of the blastozooid is formed from the posterior end of the original epicardial tube. It remains single. The neural tube arises from the left peribranchial sac.

H. fasmeriana forms a close link between the sub-family Holozoinae and the sub-family Polyclininae. It resembles the Holozoinae in form of gut, position and mode of origin of the brood pouch, and position of the cardio-pericardium. It shares with the Polyclininae the post-abdominal position of the gonads as well as the structure and organogenesis of the buds.

In the description of the embryological development of the viviparous compound ascidian *Hypsistozoa fasmeriana* (sub-family Holozoinae) (Brewin, 1956), attention was drawn to the great degree of larval budding which occurs in the species, but no detailed account of the origin and development of the larval buds was included.

Budding in the tadpole of *H. fasmeriana* conforms with that recorded for other members of the sub-family Holozoinae in that spherical buds arise from a ventral stolonic outgrowth, which contains an extension of the epicardial tube. In this species the stolon, which projects from the left side of the

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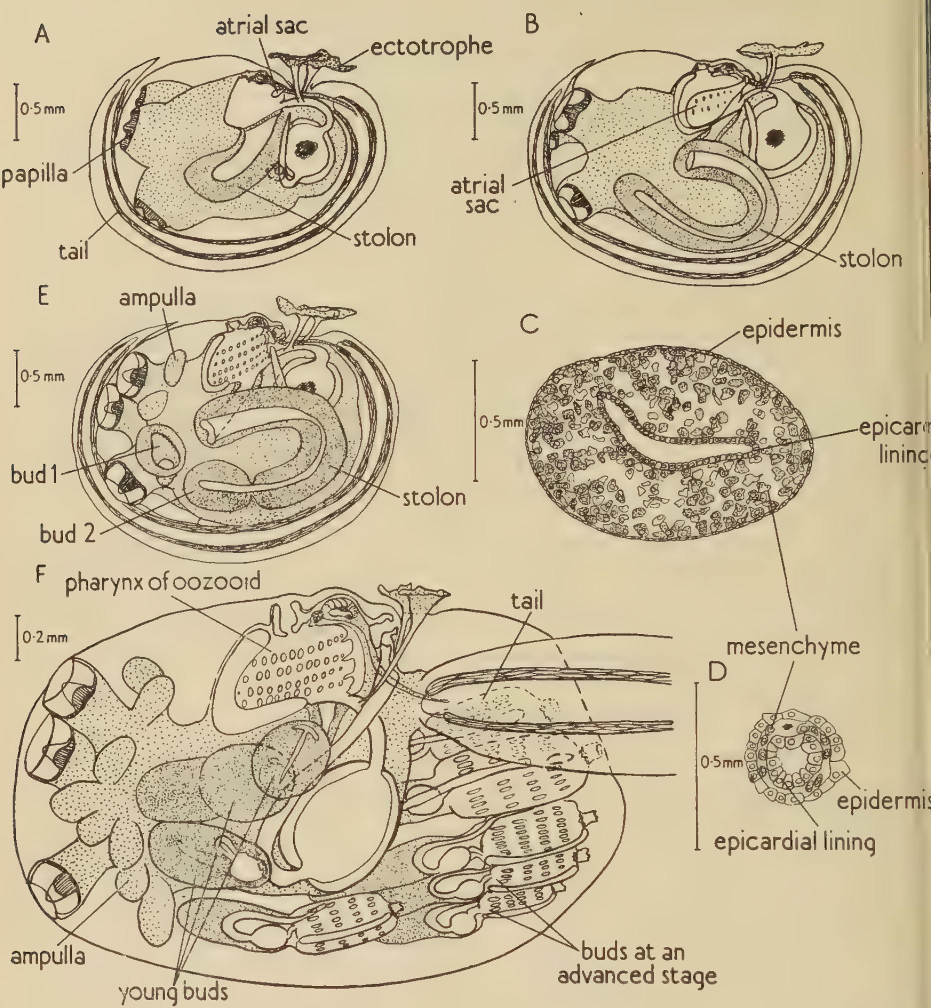

Fig. 1. Stolon and bud development of *H. fasmeriana*. A, embryo with developing stolon, ectotrophe severed close to attachment tubes, small atrial diverticula present. B, embryo with fully developed stolon. The inner walls of the atrial diverticula have fused with the side walls of the pharynx. C, transverse section of stolon of *H. fasmeriana*. D, transverse section of stolon of *D. magnilarva* (after Brien, 1939). E, embryo showing stolonial budding; one bud already cut off and another in the process of severance (ampullae appearing at the anterior end). F, a free-swimming tadpole with buds from early to advanced stages of organogenesis arranged in an arc around the oozooid (ampullae well developed).

arva at the time of atrial invagination, is approximately 235 μ in diameter. t extends rapidly, attaining a length of up to 1·8 mm. The direction of growth s at first antero-ventral for some 0·3 mm (fig. 1, A), posterior for about 0·6 mm, entral for a short distance, and finally anterior for about 0·8 mm. The resulting stolon is confined to the left side of the larva. It takes the form of a long airpin bend, of which the proximal end is attached and dorsal, the distal end ree and ventral (fig. 1, B).

The stolon consists of a thin outer epidermal layer, a thick, densely packed mesenchymal layer of cells containing numerous granules, and a thin inner picardial layer which encloses an epicardial cavity (fig. 1, C).

Severance and movement of the buds

Budding does not occur until the stolon has reached its maximal length. The buds are approximately 235 μ in diameter. They develop one at a time rom the distal end of the stolon, the initiating epidermal furrow of the second bud not appearing until after complete severance of the first bud and so on fig. 1, E). When the entire stolon has undergone constriction, 9 to 14 buds re present.

Buds are constricted off by localized activity of the epidermal layer of the tolon. The regions of the stolonic epidermis responsible for severance of buds are responsible also for a considerable amount of test secretion. Thus, y the time the second severing furrow has appeared in the stolonic epidermis, the first-formed bud has been moved away from the distal end of the stolon and is separated from it by test up to 50 μ in thickness (fig. 1, E). With secretion of test associated with the formation of each new bud, the older buds take up positions farther and farther from their original location.

Owing partly to the curved nature of the stolon from which they are derived and partly to the configuration of the oozooid, the buds follow the arc of a spiral which runs from the left side of the oozooid somewhat anteriorly across the ventral side and posteriorly up the right side (fig. 1, F). Thus in a normal larva at the end of bud formation the oldest (the first-formed bud) has been moved the farthest and is the most posterior, lying high up on the right side of the oozooid, and the youngest (the last-formed) has been moved the least and lies on the left side of the oozooid close to the point of origin of the stolon (fig. 1, F).

Orientation of the buds

The epicardial layer of the stolon remains passive until just before the actual severance of a bud (fig. 1, E). At the severed end of the bud the epicardial wall lies close to the epidermis (fig. 4, A), whereas at the other end it is separated from the epidermis by mesenchyme several cells deep. (These are either, as in the first buds, cells that were present originally at the distal end of the stolon, or, as in later buds, cells that have moved in to the new distal end of the stolon during the time-lapse between the separation of a bud and the development of the next stolonic furrow.) Thus the ends of the bud are differentiated at the time of constriction.

During subsequent blastozooid formation it is apparent that both antero posterior and dorso-ventral polarities are established at the time of bud formation. The newly severed end of a bud (that which was the more proxima of the stolon) becomes the anterior end of the blastozooid and the opposite end (that which was the more distal of the stolon) becomes the posterior end The region of the bud facing the oozooid becomes the dorsal surface of the blastozooid; the region farthest from the oozooid and nearest the periphery of the tadpole becomes the ventral surface. The larval bud also possesses an axial organization with the greatest potential for growth at the anterior end The effect of the latter is seen in the greater rate of expansion and differentiation of the anterior end compared with that of the posterior end. This axial organization may be due to some gradient of potential present in the stolon o the tadpole from which the bud was severed and/or to a new potential initiated by the mechanical stimulus of constriction.

One effect of the peculiar form of the stolon is that while buds formed from the ventral portion of the hairpin bend are so orientated at the time o severance that their anterior ends lie towards the posterior region of the tadpole, those formed from the dorsal portion are orientated in the opposite direction. Nevertheless, in a normal tadpole all buds come to lie with their anterior ends directed towards the posterior end of the tadpole (fig. 1, F) Therefore buds arising from the dorsal portion of the stolon must be involved in movement through 180° as well as in movement along the spiral pathway In some tadpoles buds arising from the ventral end of the stolon fail to accomplish this 180° movement and move along arcs other than the arc o the first-formed buds.

Fate of the larval buds in the tadpole

Each bud is a definitive bud destined to become a blastozooid. Shortly after the bud has been severed from the stolon organogenesis begins, and epicardial, pharyngeal, and atrial regions are distinguishable in the first bud before the third bud is isolated. By the time the tadpole is ready to become free-swimming the buds are clearly recognizable as blastozooids. In a normal tadpole together with the oozooid, the larval buds form a ring of zooids neatly arranged with their dorsal surfaces towards the centre and their ventral surfaces towards the circumference (fig. 1, F).

The free-swimming period of the tadpole is usually short, 5 to 35 min Fixation is followed (sometimes within 5 min) by the buckling up of the tissues

FIG. 2. Tadpole metamorphosis. A, tadpole, shortly after fixation by adhesive organs, showing retraction of notochord and surrounding structures in tail and puckering up of the test in the tail region. Blastozooids at an advanced stage of development. B, tadpole at a later stage showing test of tail released and hanging as a ghost tail; ampullae expanding to take over function of fixation. C, tadpole 2 days after fixation. Note extension and branching of ampullary processes and the arrangement of the oozooid and blastozooids. D, tadpole 12 days after fixation. Colonial form has been assumed with elongation and secretion of test by the vascular processes of the zooids and development of a common cloacal aperture; oozooid functioning along with the blastozooids. E, a two-headed colony, the oozooid and 4 blastozooids forming one head, 7 blastozooids forming the other.

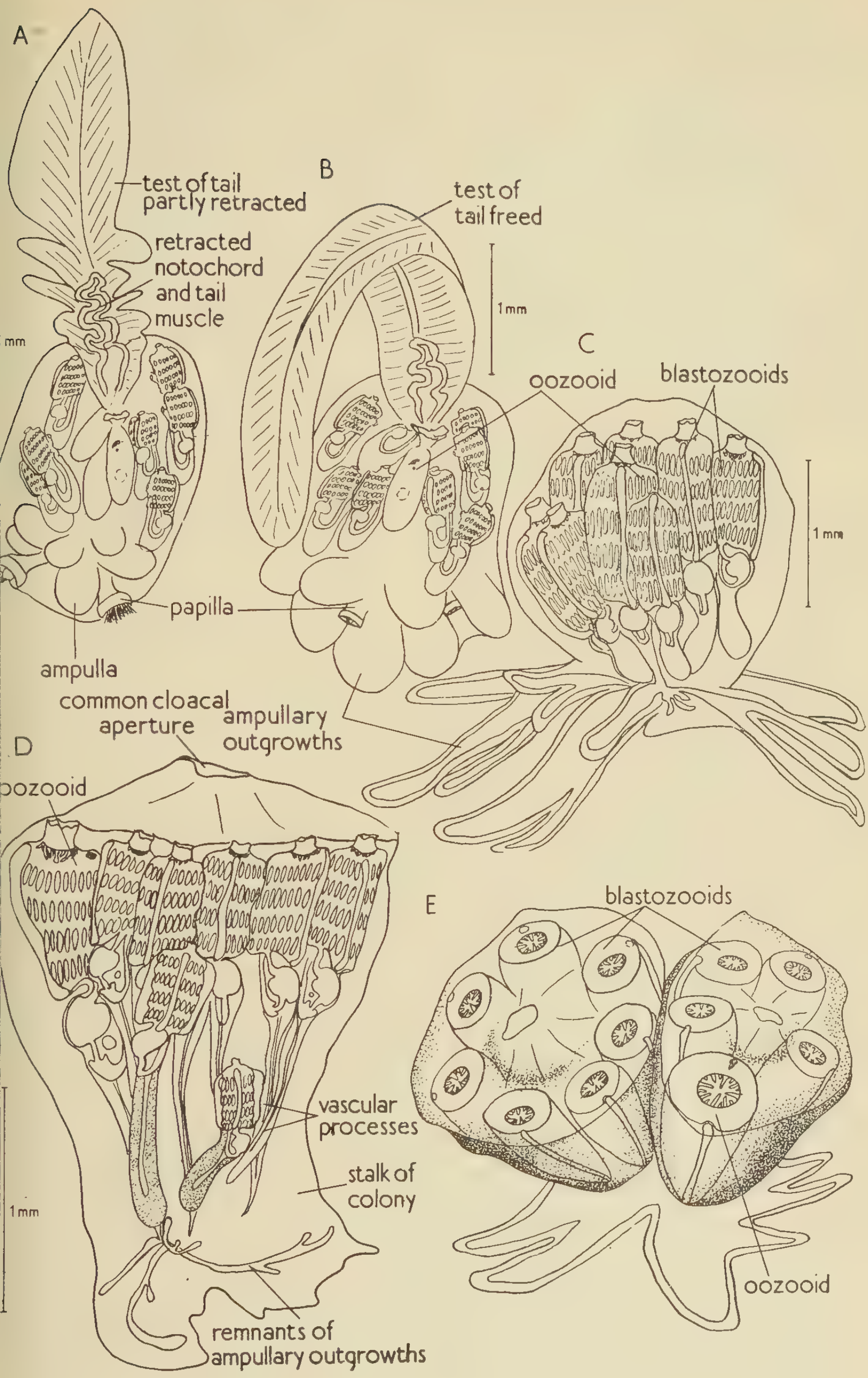
A
test of tail partly retracted
retracted notochord and tail muscle
mm
papilla
ampulla
B
test of tail freed
1 mm
C
oozooid
blastozooids
1 mm
ampullary outgrowths
common cloacal aperture
D
ozooid
vascular processes
stalk of colony
1 mm
remnants of ampullary outgrowths
E
blastozooids
oozooid

FIG. 2

of the tail (fig. 2, A) and (within 1 to 4 h) by expansion of the 9 to 14 larv ampullae. The latter secrete test and serve as the second line of attachment the tadpole. At about the same time as they are making direct contact with th substratum, the test becomes freed from the remaining tissues in the ta region and resumes its original length, hanging like a ghost tail (fig. 2, B) unt it becomes detached and is wafted away. The ampullae reach a length of up to mm and together with their side branches form an imposing anchorage syste (fig. 2, C). During the period of ampullary attachment the buds show consider able increase in size (fig. 2, C). Growth of the anterior pharyngeal regions of th blastozooids brings their anterior ends nearer the outer surface of the test (fig. C, D); growth of the posterior regions results in the formation of long slende vascular processes (fig. 2, D). With extension of the latter, together with te secretion by their epidermal coverings, a definite stalk region is establishe and the metamorphosed tadpole assumes the colonial form shown in fig. 2,

Five days after fixation the test ruptures in the areas overlying the branchi apertures of the oozooid and of the blastozooids. In each zooid the cilia the pharyngeal region are beating actively at this time. The central regio of the test ruptures and a common cloacal aperture is formed in the centr of the head of the young colony.

After metamorphosis each tadpole becomes a small colony, usually single headed (fig. 2, D), but more rarely two- or three-headed (fig. 2, E). The numbe of heads depends upon the manner in which the groups of blastozooid derived from the larval buds are orientated, and this in turn depends upon th movement of the larval buds. From tadpoles in which all the larval buc follow the spiral pathway taken by the first-formed buds and form a singl ring of zooids, a single-headed colony develops. From tadpoles in which som of the buds move along arcs other than that followed by the first-formed buc and form two or three rings of zooids, a two- or three-headed colony develop In such colonies each head has its own ring of blastozooids, but in only or head is the oozooid incorporated in the ring (fig. 2, E).

It is of interest to note that in the tadpole the larval buds, together wit the oozooid, form a ring which is tilted slightly away from the vertical. Whe the tadpole becomes fixed by the anterior papillae and metamorphosis tak place, the plane of buds and oozooid is altered and the ring becomes mo nearly horizontal, still, however, retaining the tilt and with the sector contai ing the oozooid occupying the most elevated position. Thus in young coloni the plane of the head is slightly off the horizontal, an arrangement which pe sists throughout the life of the colony, i.e. the imprint of larval form laid upo the young colony is a decisive factor throughout the entire life of the colon

Fate of the larval buds in the colony

In *H. fasmeriana* 5 days after fixation of the tadpole the oozooid functio *simultaneously* with the nine or more blastozooids of the first generation (i. those derived from the larval buds given off by the stolon of the tadpole) ar moreover the oozooid *continues to function for 6 weeks or more.*

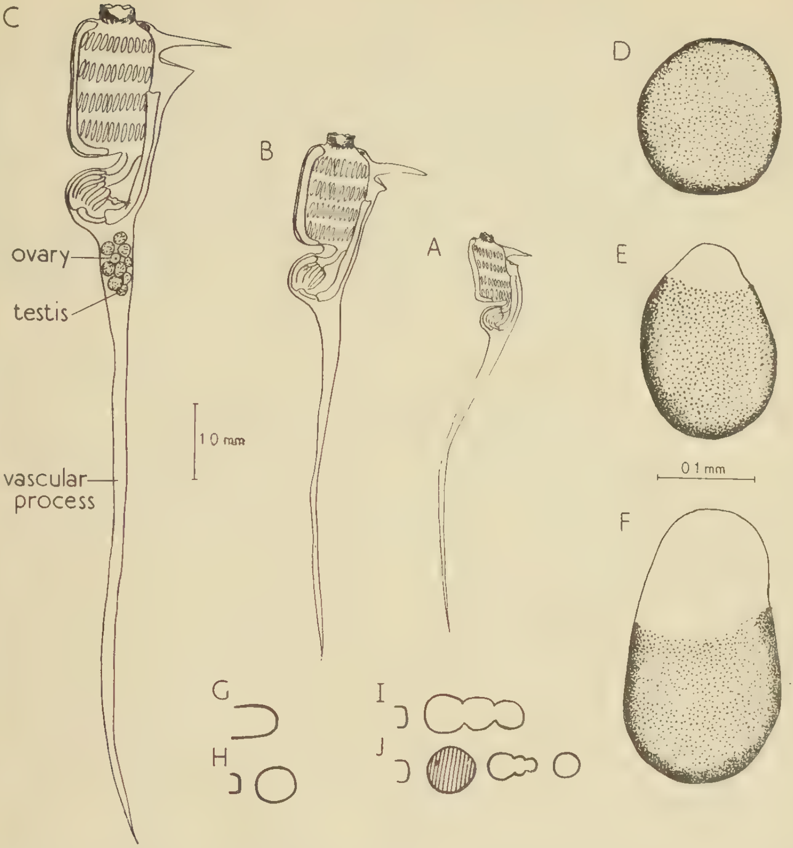

FIG. 3. A, B, C, blastozooids of *H. fasmeriana* from colonies collected on 21 May 1954. All are drawn to the same scale. A, blastozooid of the first generation from a colony 5·3 mm in height, established November 1953. B, blastozooid from a colony 8 mm in height, established November 1952. C, blastozooid from a colony 12 mm in height, established November 1951. D, E, F, larval buds of *H. fasmeriana* showing the shape and general appearance during organogenesis, at Stages I, II, and III respectively. G, H, I, J, diagrammatic representation of larval budding in the genus *Distaplia*. G, the stolon before budding commences. H, the single stolonic bud. I, the probud, the result of development of the stolonic bud. J, buds resulting from severance of the probud. Cross-shading indicates a definitive bud, that is, one destined to become a blastozooid.

It was noticed that in the few tadpoles of *H. fasmeriana* in which one or two buds were not as advanced as the rest at the time of metamorphosis, such buds developed rapidly during the first few weeks of colonial life and their development was not delayed until extra nourishment became available through dedifferentiation of the oozooid (fig. 2, D).

The blastozooids of the first generation reach a length of approximately 5 mm. With continued histolysis of the mesenchyme they become transparent throughout and though gonadial tissue possibly may be present in larval buds at the time of their inception, it has not been recognized at any stage in the blastozooids of the first generation (fig. 3, A).

Attainment of sexual maturity by the blastozooids

Within the colony numerous generations of blastozooids arise. The blasto zooids of each succeeding generation are larger than those of the precedin generation, until maximal size is reached (fig. 3, A, B, C). It is only afte colonies have been established for 2½ years that the blastozooids approximat 10 mm in length and become sexually mature (fig. 3, C). Such blastozooid are protandrous hermaphrodites with testes discernible from early in Ma until the end of July. The single ovum reaches maximum developmen towards the end of May (Brewin, 1956).

Comparison of the larval budding of Hypsistozoa fasmeriana *with that of othe members of the sub-family Holozoinae*

Only three genera are known in this sub-family: *Distaplia* Della Vall (1881), *Sycozoa* Lesson (1830), and *Hypsistozoa* Brewin (1953).

In the genus *Distaplia* buds have been recorded in tadpoles of *D. magnilarva* *D. rosea*, *D. bermudensis*, *D. garstangi*, *D. taylori*, *D. knoxi*, and *D. stylifera* They are also present in *D. japonica* and *D. occidentalis* (author's collection) In the genus *Sycozoa* buds are known to be present in the tadpoles of on species only, *S. tenuicaulis* (Brewin, 1953). In neither genus, however, is th degree of larval budding so extensive as it is in the genus *Hypsistozoa*.

Larval budding follows a similar pattern in *Distaplia* spp. and *S* *tenuicaulis*. The budding in *D. magnilarva*, the species in which it has bee most closely investigated, can be taken as typical of that of the two genera The initiating stolon, which projects from the left side of the oozooid, though much smaller than that of *H. fasmeriana*, has the same basic structure. Th only major difference is in the mesenchyme, the cells of which in *D. magnilarv* are few and not heavily granulated (fig. 1, D) (Brien, 1939).

In *D. magnilarva* and *D. bermudensis* the severance of a bud by epiderma constriction starts almost as soon as the stolon projects from the oozooi (Della Valle, 1881; Brien, 1939). In *D. magnilarva* one stolonic bud only i produced and no further development of the stolon occurs. In *D. bermudensi* growth of the stolon continues after the severance of the first stolonic bud an another—a small residual bud—is later cut off (Berrill, 1948). This is unlik the condition in *H. fasmeriana* where the stolon reaches maximal size befor any severance occurs.

Berrill (1948) has shown that at the time of severance of the larval buds o *D. magnilarva*, antero-posterior and dorso-ventral polarities are established These polarities are identical with those of the larval buds of *H. fasmeriana* The axial organization also is similar to that of larval buds of this species, th greatest potential for growth being at the anterior end.

Whereas the buds of *H. fasmeriana* may be 235 μ in diameter, in *Distaplia* and *Sycozoa* the newly severed stolonic buds are small, being either (i approximately 45 μ in diameter (*D. magnilarva*, *D. rosea*, *D. bermudensis*

. *taylori*, *D. knoxi*, *D. garstangi*, and *S. tenuicaulis*), or (2) approximately 15 μ diameter (calculated for the residual bud of *D. bermudensis* from fig. 16, errill, 1948). Buds of the latter size presumably become abortive owing to eir small initial size (Berrill, 1948). The buds of approximately 45 μ in iameter, lying freely in the test of the tadpole, show a marked increase in ze (fig. 3, H, I). The length becomes 3 or 4 times the original length, and the iameter, the main increase in which is at the anterior end, becomes approxiately 1¼ times the original diameter (fig. 3, I). In its elongated form the tolonic bud is known as a probud. Growth of the probud is accompanied y a slight amount of morphogenesis. Brien (1939) notes in the probud f *D. magnilarva* doubling of the epicardial component both anteriorly and osteriorly and formation of a dorsal cordon. Berrill (1948) describes in the robud of *D. bermudensis* doubling of the epicardial component by invagination long its main axis and the formation of a solid mass of 'reproductive and ther mesodermal tissue' from one half of the epicardial tissue.

The probud undergoes division into 3 almost spherical portions, severance aking place by epidermal constriction (fig. 3, J). Of the portions resulting rom division of the probud, the anterior one is the largest and most highly rganized, the central one is smaller and less highly organized, and the osterior one is the least highly organized. The fate of the portions of the robud varies. The posterior portion may become abortive or develop into probud which in turn divides (*D. magnilarva*, *D. rosea*, *D. japonica*); the entral portion may become abortive, may develop into a probud (*D. magniarva*), or may become a definitive bud capable of developing into a blastoooid; the anterior portion, which is larger than the initial larval bud, beomes a definitive bud capable of developing into a blastozooid (*D. rosea*, *. magnilarva*). The larger stolonic buds of *H. fasmeriana* all become definiive buds and develop into blastozooids. The smaller stolonic buds of *Distaplia* nd *Sycozoa* do not become definitive buds directly. A probud stage interenes, in which increase in size occurs. It is only after strobilation of the robud that a bud is formed which is larger than the initial bud and of dequate size for development into a blastozooid.

In *Distaplia* and *Sycozoa* species the stolonic buds and the portions of the robuds derived from them remain on the left side of the larva close to its entral surface. What little movement there is, is in an anterior direction. hese buds being few and small occupy a niche that would be too restricted or the larger and more numerous buds of *Hypsistozoa*.

In *H. fasmeriana* by the time the tadpole becomes free-swimming the buds ave attained an advanced degree of morphogenesis. In *Distaplia* and *Sycozoa* pecies by the time the tadpole becomes free-swimming even the largest buds ave achieved only a lowly stage of development.

In the genus *Distaplia* the best account of the young colony is given by errill (1948), who states that in *D. rosea* 5 days after fixation of the tadpole he oozooid functions *alone* and continues to function for 16 to 24 days. Then edifferentiation of the oozooid occurs and *after this* one blastozooid *only* of the first generation becomes fully organized and acts as a functional uni (Berrill, 1948). The form of the colony in *D. rosea* is much more haphazar than in *H. fasmeriana* and the arrangement of the larval buds makes n imprint on the form of the young colony.

Gonadial tissue is not discernible in the larval buds of *H. fasmeriana* an would appear to be variable in appearance in *Distaplia* species. Berrill (1939) in summing up the presence of gonadial tissue in both colonial and larval bud of the sub-family Holozoinae, remarks: 'The variable quantity of the presumptive gonad tissue seems to be the cause of the great variability in th nature of the gonads subsequently developed. The buds produced by th oozooid do not exhibit any discernible sign of gonads of either sex whe fully developed, in the case of *D. rosea*, but early stages show rudimentar gonads of either sex though never both together . . . mature ovaries develop only when the presumptive gonad initially segregated is quantitativel adequate. Sub-maximal masses may well give rise to main components only as in *D. magnilarva*.' It is concluded that the larval buds of *H. fasmeriana* ar of sub-maximal size for the species, since the blastozooids of the first generation are asexual with the main components only developing.

In the whole sub-family Holozoinae the tadpoles produced bear a clos resemblance to one another. Hence it is reasonable to suppose that, of th energy available to the embryo, only that surplus to what is required for th production of the free-swimming tadpole will be available for the development of buds.

Species of the genus *Distaplia* produce yolky eggs ranging from 290 μ t 520 μ in diameter. In the tadpoles developed from these eggs only one or tw small larval buds are formed.

Species of the genus *Sycozoa* produce yolky eggs from 280 μ to 370 μ in diameter. Larval budding is recorded in *S. tenuicaulis* only and in this species only two small buds are formed (Brewin, 1953).

H. fasmeriana, the only known species of the genus *Hypsistozoa*, has minute, alecithal egg 25 μ in diameter (Brewin, 1956). The species is viviparous and the amount of energy obtained from extra-embryonic sources is sufficient for the production of a tadpole much larger than any other in the sub-family and also for the development in the oozooid of a large epicardial stolon capable of producing 9 to 14 large larval buds.

It is concluded that in the larval stage (1) the size of the primordial bud, i.e. that derived directly from the stolon, is determined by the diameter of the stolon, which is in turn determined by the amount of energy available for the developing embryo, and (2) the fate of the primordial bud is determined by its initial size. In *Distaplia* species the larval bud becomes a probud, which, free in the substance of the test, increases in size before it strobilates with the production of at least one bud which is larger than the original stolonic bud and of adequate initial size for development as a definitive bud. All the larval buds of *H. fasmeriana* are of adequate initial size for development as definitive buds.

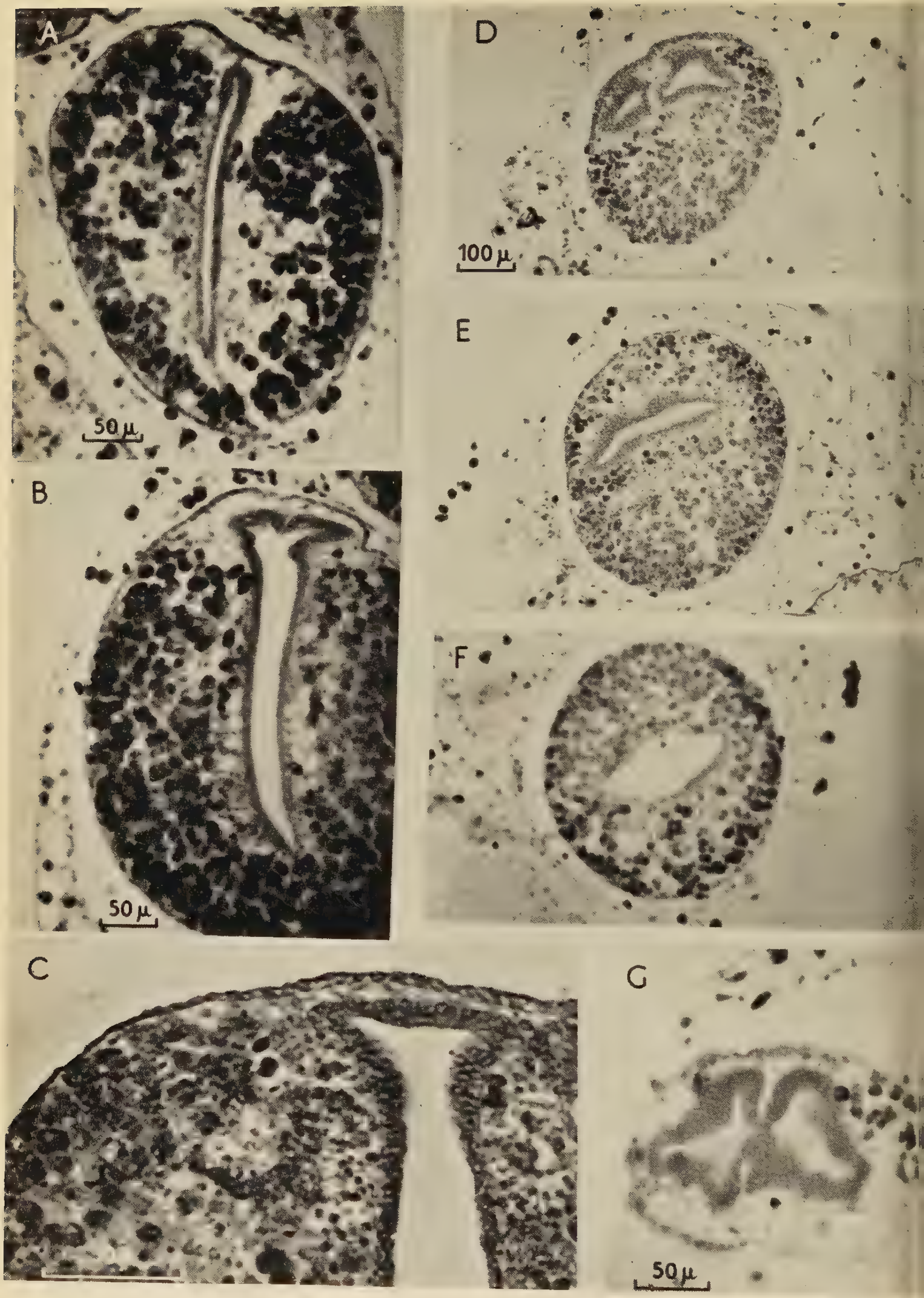

FIG. 4

B. I. BREWIN

Organogenesis of the larval buds of Hypsistozoa fasmeriana

The larval bud of *H. fasmeriana* at its inception is completely opaque, wing to the presence of numerous granulated mesenchyme cells which lie etween the outer ectodermal and the inner epicardial epithelia (fig. 3, D). nternally the wide, rather shallow, median epicardial cavity occupies the nterior two-thirds or three-quarters of the bud (fig. 4, A). Both epicardial and ctodermal epithelia are of pavement type except at the end newly severed—he future anterior end of the bud. Here the epicardial cells are cubical or olumnar in form; the ectodermal cells are more cubical in form and have lenser contents than the other ectodermal cells (fig. 4, C).

Organogenesis of the bud is at first rapid during formation of the main rgans and then slow during a period of growth and elaboration. For con-enience of description 5 stages in organogenesis are recognized.

Stage I is a period of elaboration of the epicardial epithelium, with two listinct but very short lobes forming at the anterior end (fig. 4, B. D. E, F).

Stage II is a period of elongation, during which the extreme anterior end f the bud pushes out as a narrow clear outgrowth (fig. 3, E). Internally the wo anterior lobes, which were differentiated at Stage I, increase in length and ecome crescentic in transverse section (fig. 5, A). Close to the point of origin f the lobes the epicardial epithelium approximates dorso-ventrally and ellular growth at this point results in the formation of a narrow bar of tissue hich traverses the epicardial cavity of the bud (fig. 4, G). This bar virtually eparates the two anterior lobes—the future pharyngeal region—from the ingle posterior region—the epicardium proper. The pharyngeal region emains in communication with the epicardium by wide openings (fig. 5, C), ne on the right and one on the left of the narrow bar of tissue. At the same me as the latter is developing a very short diverticulum—the rudiment of the ut loop—arises from the wall of the right anterior lobe (fig. 5, A).

In *Stage III* also, elaboration and growth is confined mainly to the anterior nd. In this period the bud becomes more truly ovoid in shape (fig. 3, F). nternally the two anterior lobes fuse to form a single pharyngeal region. The ndostylar rudiment appears as a thickening of the ventral wall of the pharynx.

FIG. 4 (plate). Photomicrographs of early stages of organogenesis of the larval buds of *. fasmeriana*. Material fixed in Bouin, stained with borax carmine, and sectioned at 8 μ.

A, longitudinal section through a newly severed bud, showing ectoderm, mesenchyme, and icardium.

B, longitudinal section through a bud at a slightly later stage, showing at the anterior end e doubling of the epicardial component.

C, longitudinal section through the anterior end of a newly severed bud, showing that at is end the epicardial cells are columnar in form and that the central ectodermal cells are ore cubical than elsewhere.

D, E, F, transverse sections through one bud, showing in D the two anterior lobes of the icardial component, in D and E the columnar form of the epicardial cells, and in F the pave-ent form of the epicardial cells of the posterior region of the bud.

G, transverse section through a bud at the point at the posterior end of the epicardial lobes here the epicardial lining invaginates dorsally and ventrally. This invagination brings about e development of a bar of tissue which virtually separates the two anterior lobes (the future aryngeal region) from the epicardium proper.

Two peribranchial sacs arise, right and left, as outpushings of the latero-posterior walls of the pharynx. These sacs extend rapidly around the dorsal side of the pharynx and fuse to form a single atrium. The neural tube arises as a tubular outgrowth projecting forward from the left peribranchial sac (fig. 5, F, E, D). During this stage the gut rudiment pushes out in a postero-ventral direction as a straight hollow outgrowth. The epicardium proper remains single, but extends posteriorly; growth is unequal and the left side becomes more extensive than the right.

Stage IV is a period of general elongation distinguished by development of the cardio-pericardium and elaboration of the alimentary canal.

The rudiment of the cardio-pericardium arises from the union—just posterior to the gut loop—of two small ventral outgrowths of the epicardium—one from the right side and one from the left. At first the cardio-pericardium retains communication right and left with the epicardium proper (fig. 6, A, B, C, D), but, within a very short time, development of lateral longitudinal constrictions, right and left, brings about its isolation. The cardiac region proper forms as an infolding of the dorsal wall.

Elongation of the gut diverticulum results in the formation of a blind U-shaped tube with a small lateral diverticulum, the rudiment of the pyloric gland, developing on the inner side of the descending limb.

In the pharyngeal region towards the end of Stage IV the endostylar groove is well developed; a forward prolongation has occurred in the region of the future branchial aperture; and cellular contacts have been made between the lateral walls of the pharynx and the inner walls of the peribranchial sacs (fig. 6, A).

Stage V is a lengthy stage in which advanced morphogenesis is attained. Histolysis of the granulated mesenchyme is rapid during this stage.

Changes in the pharyngeal region include formation of the gill slits, development of the branchial tentacles, and establishment of the neural complex. The dorsal wall of the neural tube becomes considerably thickened and a portion of the thickened area separates off as the nerve ganglion. The posterior end of the neural tube loses its connexion with the left peribranchial sac, while near the anterior end communication is made with the wall of the

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FIG. 5 (plate). A, B, transverse sections cut at 8 μ through a bud at Stage II.

A, a section from the anterior end showing the crescentic form of the anterior lobes of the epicardium and the origin of the gut rudiment from the right anterior lobe.

B, a section cut slightly behind A, showing the formation of the bar of tissue at the posterior end of the two anterior lobes.

C, median sagittal section of a bud at Stage III, showing the dorso-ventral bar of tissue which separates the pharyngeal region from the epicardium proper. The unequal elongation of right and left sides of the epicardium proper is also shown.

D, E, F, transverse sections of a bud from a series cut at 8 μ, showing the origin and structure of the neural tube.

D, the twelfth section from the anterior end of the bud. The neural tube lying between the right and left peribranchial sacs exhibits a distinct thickening of the dorsal wall.

E, the fourteenth section from the anterior end of the bud. The neural tube lies between the right and left peribranchial sacs and the wall shows no differentiation.

F, the fifteenth section from the anterior end of the bud, showing the neural tube arising from the left peribranchial sac.

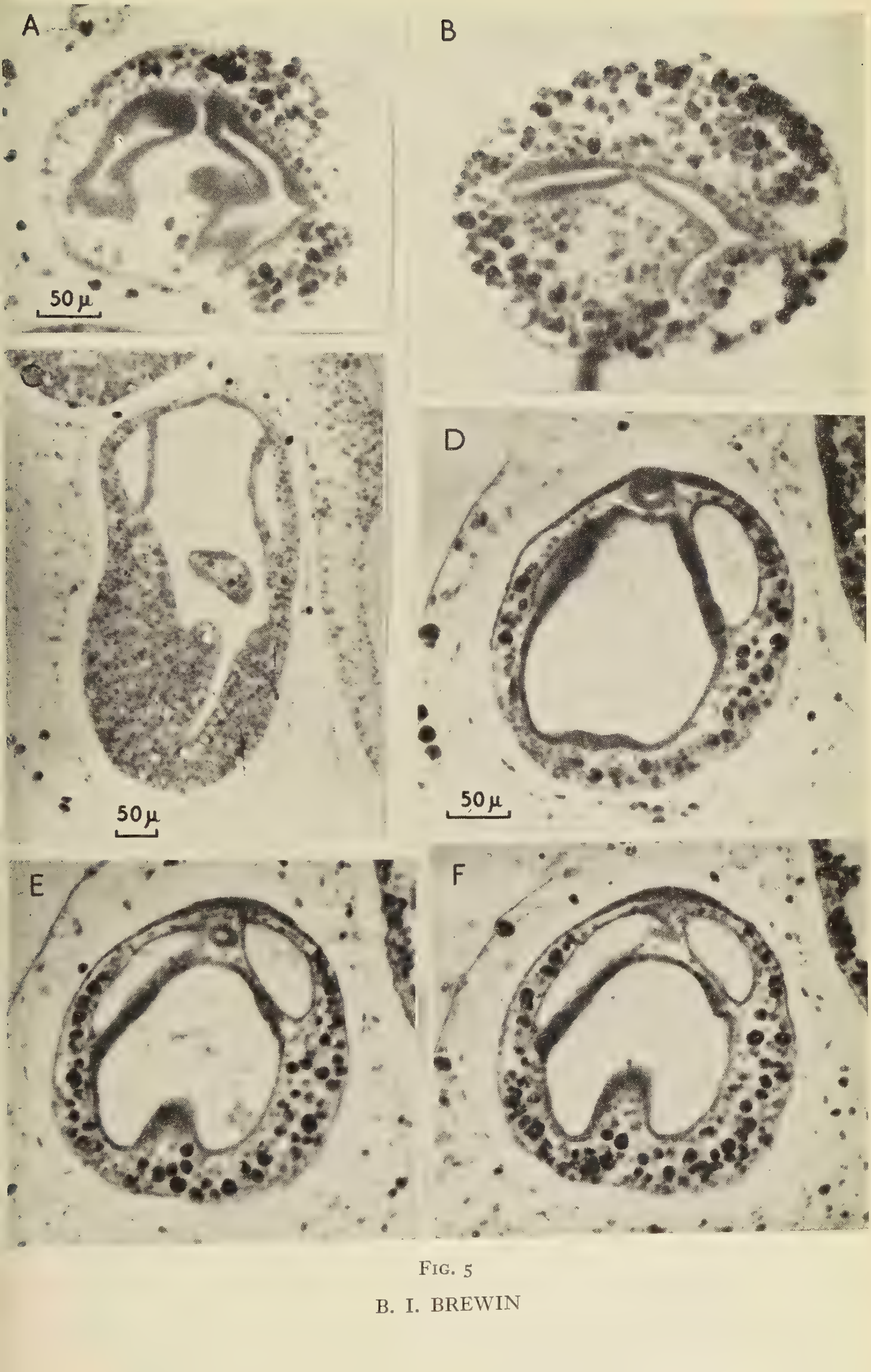

FIG. 5

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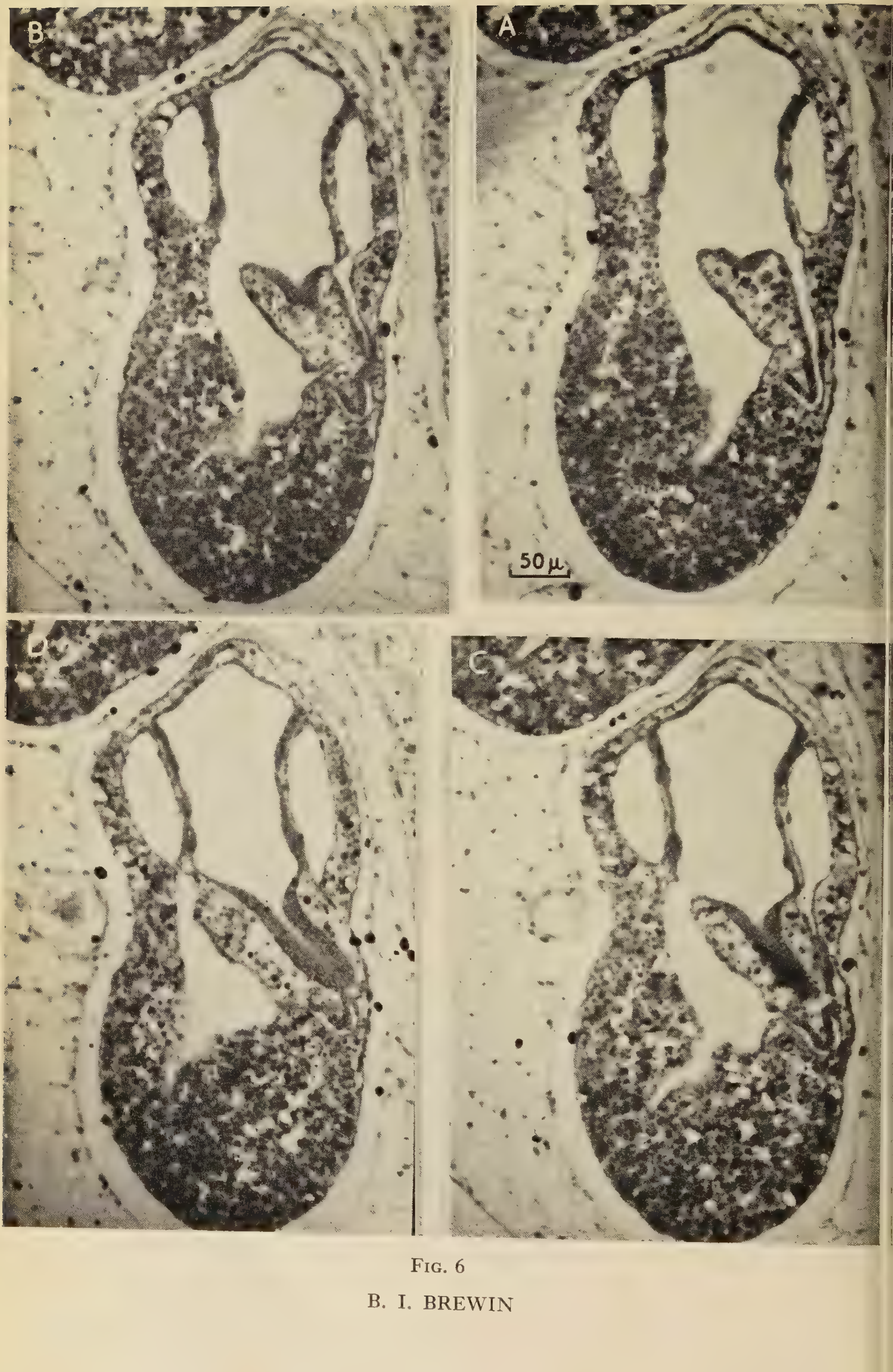

Fig. 6

B. I. BREWIN

harynx and the neuro-hypophysial complex (ciliated funnel, dorsal tubercle, nd neural gland) is established.

Changes in the gut loop result in the establishment of the various regions—esophagus, stomach, and intestine. Extension of the pyloric diverticulum ccurs. The distal end fuses with the ascending limb of the intestine, and a yloric reservoir develops in the central region. During this stage the anus reaks through into the atrial cavity and the atrium itself extends out in the egion of the future atrial aperture, pushing the surmounting ectodermal layer vith it.

Developments in the epicardial region are further posterior extension on he left side and constriction of the pharyngeal-epicardial connexions right nd left.

Comparison of organogenesis in Hypsistozoa fasmeriana *with that of other members of the sub-family Holozoinae*

Within the sub-family Holozoinae organogenesis of the larval buds has een described also in various species of *Distaplia*. The epidermis of the larval uds never contributes anything except epidermal tissue and structures, vhereas the inner epicardial lining gives rise to all the internal organization, ncluding the nervous tissue, the heart, and in most cases the gonads. The lifferences in organogenesis in the two genera are slight and lie mainly in the rigins of the epicardium proper, the pharynx, the neural tube, and the gonads.

In *H. fasmeriana* the epicardial region of the initial bud is extensive and the osterior end of it becomes separated off as the epicardium of the blastozooid. This, the epicardium proper, is single from the time of inception of the bud. n the various species of *Distaplia* the epicardial region of the initial bud is hort and in most species the epicardium proper is double for at least part of ts existence. In *D. magnilarva* a medio-sagittal furrowing separates the osterior end of the original epicardial cavity into right and left portions Salensky, 1893; Brien, 1939; Bonnevie, 1896). Bonnevie notes that the urrowing does not extend to the extreme posterior end and that the picardium proper in the blastozooid takes the form of a U. In *D. rosea* he epicardium proper is said to arise as an outgrowth from the posterior end f the epicardial tube (Berrill, 1948).

A doubling of the anterior end of the epicardial tube before formation of he pharynx, similar to that which occurs in *H. fasmeriana*, is recorded by

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FIG. 6 (plate). Photomicrographs of a series of sagittal sections cut at 4 μ through a bud t Stage IV to show the relationship of cardio-pericardium and epicardium.

A, the thirty-first section of the series, showing the diverticulum from the right side of the picardium, which enters into formation of the cardio-pericardium.

B, the thirty-second section of the series, showing the cardio-pericardium lying among he mesenchyme cells.

C, the thirty-fourth section of the series, showing the diverticulum from the left side of the picardium, which enters into formation of the cardio-pericardium.

D, the thirty-fifth section of the series, showing the position of the heart just posterior to he gut loop.

Brien for *D. magnilarva* (Bonnevie makes no mention of it). Likewise Berri (1948) makes no mention of it in *D. rosea.*

In the sub-family Holozoinae the origin of the neural tube as an extensio of the left peribranchial sac is characteristic of *H. fasmeriana* only. In th genus *Distaplia* records show that it arises as a direct outgrowth of the inne vesicle either in the probud stage as part of a dorsal cordon (Brien, 1939) o at a later stage as an independent outgrowth of the inner vesicle (Bonnevie 1896; Berrill, 1948).

The presence or absence of gonadial tissue varies in larval buds even withi the one species. In species of *Distaplia* it is recorded as being present in th larval bud at its inception (Bonnevie, 1896), as arising in the probud stage a part of the dorsal cordon (Brien, 1939), and as arising from a region severe off the original inner vesicle (Berrill, 1948). In larval buds of *H. fasmeriana* n gonadial tissue is discernible.

Comparison of the larval buds of Hypsistozoa fasmeriana *with buds of the sub-family Polyclininae*

The larval buds of *H. fasmeriana* resemble the buds of the sub-famil Polyclininae (family Polyclinidae) in the possession of much heavily granulate mesenchyme and of an extensive epicardial tube, the posterior end of whic becomes the epicardium proper of the blastozooid.

A further similarity lies in the fact that the origin of the neural tube fro the left peribranchial sac, which distinguishes organogenesis of the buds o *H. fasmeriana* from that of other members of the sub-family Holozoinae, i characteristic also of the sub-family Polyclininae.

Systematic position of Hypsistozoa fasmeriana

H. fasmeriana stands in an intermediate position between the sub-family Holozoinae and the sub-family Polyclininae, sharing with the Holozoinae the form of the gut, the position and mode of origin of the brood pouch, and the structure and position of the cardio-pericardium (Brewin, 1956), and with the Polyclininae the post-abdominal position of the gonads and the structure and organogenesis of the buds.

After a study of epicardial development Brien (1929) concluded: 'Nous pouvons donc nous représenter les Polyclinidae comme dérivant des Polycitoridae [= Holozoinae] dont ils représentent une forme évoluée aberrante.' In the classification of the Ascidiacea, Berrill (1950) places the sub-family Holozoinae as the highest of the family Clavelinidae and the sub-family Polyclininae as the lowest of the next family, the Polyclinidae.

With the discovery of *H. fasmeriana* further weight is added to the views postulated above and an even closer link is forged between the families Clavelinidae and Polyclinidae.

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An Improved Radioautographic Method for demonstrating Tyrosine Uptake and Tyrosinase Activity in Melanocytes

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With one plate (fig. 1)

SUMMARY

A method for studying the melanogenetic activity of pigment cells is described. Tissue is incubated in a medium containing tyrosine labelled with ^{14}carbon. Tyrosine, being a precursor of melanin, is incorporated into the insoluble pigment. This can be detected in histological selections by radioautography. A measure of tyrosine uptake and, provided precautions are taken, of tyrosinase activity, can be gained by the use of this technique. There are advantages in using labelled, rather than 'cold' substrate.

This technique, developed by other workers, has been used with success; but difficulties arise in situations where intense pigmentation obscures the image of the superimposed radioautograph. Here consideration is given to the possible methods for circumventing this difficulty, and a satisfactory method for partially bleaching the pigment, without upsetting the radioautograph, is described in detail.

IT is now generally accepted that the pigment melanin is derived from tyrosine by a process involving oxidation, both enzymic and spontaneous, and polymerization. It is notable that the precursor, tyrosine, is water-soluble while the pigmented product is totally insoluble, a fact that has been made use of by Kukita and Fitzpatrick (1955) in developing a method for investigating the rate of pigment synthesis by melanocytes in various organs (both normal and neoplastic) in a range of animals at different times in their life-histories (summarized in Fitzpatrick, Brunet, and Kukita, 1958).

In principle, the method of Kukita and Fitzpatrick (1955) is as follows. Tissue, containing melanocytes, is incubated in a solution of tyrosine isotopically labelled with ^{14}carbon. If the melanocytes are active, tyrosine is taken up and converted to the insoluble pigment. Excess of free tyrosine is washed out after incubation, then sections of the tissue are prepared. Radioactivity in the sections, due to uptake and conversion of tyrosine to melanin, s detected and located by radioautography (where the degree of radioactivity s indicated by the number of silver granules present in a sensitive film that overlies the tissue section). By this means it has been shown that melanogenesis is generally a sporadic, and not a continuous, process occurring at definite times in particular organs.

On the supposition that tyrosine can be incorporated not only into melanin, but also into components of tissue other than melanin, it is necessary to make adioautographs that will demonstrate the extent to which this is taking place.

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Control radioautographs must therefore be made of tissue from an albino animal given exactly the same treatment as the pigmented tissue under investigation; or radioautographs may be made of pigmented tissue incubated in a solution of tyrosine together with an inhibitor of tyrosinase such as diethyldithiocarbamate, which serves to block the copper atoms of the enzyme, thus inactivating it, or 4-chlororesorcinol, which is a competitive inhibitor. A positive radioautograph under these circumstances would indicate incorporation of tyrosine into material other than melanin. In practice it is found that the uptake of tyrosine under these circumstances is incomparably less than that which undergoes conversion into melanin.

When these control incubations are carried out, the technique is to be regarded as a highly specific, quantitative, histochemical method for the detection of tyrosinase activity. Its sensitivity is extreme: Yoshida (1959) finds it possible to detect in a radioautograph 8×10^{-10} μc/100 μ^2, which is equivalent to an uptake of some 10^{-12} g labelled tyrosine / 100 μ^2. By reason of its sensitivity the method of Kukita and Fitzpatrick (1955) is a great advance over previous methods that are based on the direct measurement of pigment deposited on the melanin granules as a result of incubation in (unlabelled) tyrosine, an estimation that becomes virtually impossible if the granules are already intensely dark (as in black hair follicles).

The reliability of the method is indicated by the comparable determinations of tyrosinase activity obtained when the radioautographic method and a manometric method are used (Miyamoto and Fitzpatrick, 1957).

Difficulties have on occasion been encountered when using this technique in cases where the density of the pigment granules has been so great as to lead to excessive local absorption of incident light. In these circumstances the microscope can form no image of the silver grains of the over-, or underlying, radioautograph: the silver grains indicating the degree of radioactivity often cannot be seen, let alone counted. To obviate this, a technique for bleaching melanin has been developed. A number of methods were tested, and one has proved very satisfactory. Melanin is bleached to a golden-yellow colour and, while the silver grains are incidentally bleached at a stage in the procedure, their blackness is restored by photographic redevelopment. Radioautographs bleached in this way are very suitable for silver grain-counting, enabling measurement of tyrosine uptake, and of tyrosinase activity, to be made.

The general procedure described here is essentially that of Kukita and Fitzpatrick (1955), but with the incorporation of the bleaching process. In addition, a different colouring agent for the histological section is employed: carmalum, which does not soften the radiographic film, is advocated; this also enables colouring to be carried out at room temperature rather than in the cold room. The use of stripping film AR 10 of Kodak Ltd. (U.K.) is recommended; this is laid on to an ordinary microscope slide, and it is very much cheaper and in some ways more convenient to use this than to use the coated slides NTB 3 made by Eastman Kodak Inc. (U.S.A.).

METHOD

The procedure described here has been found suitable for use with mam-alian skin and eye. Distilled water should be used except where tap-water specified.

Small pieces of tissue (of dimensions approximately 1 to 2 mm) are removed from a freshly killed animal and are then, without fixation, immersed in 2·2 ml of 0·1 M phosphate buffer at pH 6·8, containing 0·3 μc of DL-tyrosine-2-^{14}C, together with 3,000 units of penicillin.

The tissue is incubated in this for 24 h at 37° C and should be under constant agitation.

As a control, a similar piece of tissue is incubated in the above medium containing in addition sodium diethyldithiocarbamate at a concentration of 0·01 M. This substance inhibits tyrosinase activity, and an uptake of tyrosine in the presence of the inhibitor indicates its incorporation into structures other than pigment granules.

After incubation the tissue is fixed in 4% formaldehyde solution (10% formalin) for one hour.

It is then washed in running tap-water for 8 h.

The tissue is dehydrated, cleared, and embedded in wax.

Sections are cut at 4 μ. These are attached to microscope slides that have been coated with gelatin as described in section 10.

Microscope slides are prepared by first cleaning in a mixture containing:

potassium dichromate	100 g
sulphuric acid	100 ml
water to make	1,000 ml

After prolonged washing in tap-water, they are rinsed in distilled water.

The water is shaken off each slide, which is then dipped in gelatin solution. The slides are left to drain in a dust-proof place. So treated, the slides may be kept for several months. After a time, if kept in the light, they lose their adhesiveness.

gelatin	5·0 g
chrome alum	0·5 g
water	1,000 ml

(Add the chrome alum after the gelatin has dissolved.)

Sections of the tissue in wax are floated on distilled water at 48° C and are allowed to expand. They are lifted out on the gelatin-treated slides, which are dipped into the water below them. The sections adhere firmly to the slides without further adhesive (such as albumen).

The attached sections are allowed to dry. If necessary they may be stored in this condition (compare fig. 1, A).

When convenient, wax is removed from the dry slides with xylene; the slides are rehydrated and are taken to the dark-room in distilled water.

The following stages are carried out in the dark-room lit by a red safelight.

14. A large photographic dish filled with distilled water maintained at 24±1° (should be available. Kodak AR 10 stripping plates are used, and thes should be scored, by means of a one-edged razor blade, with lines draw at right angles so as to produce pieces of film of the right size to cover th section and to overlap the edges of the microscope slide; for one or tw sections, pieces 30 mm × 33 mm are suitable. These pieces of film ar stripped off their glass support with fine forceps and laid on the surfac of the water; the surface of the film that was adjacent to the glass suppor should be uppermost.
15. The film is allowed to expand on the water surface for 2 min. A slide i then dipped into the water below the floating film, which is then lifted ou on the surface of the slide.
16. The slide, bearing the section, now covered over with sensitive film, i drained of surplus water and set in a rack to dry.
17. Drying is best carried out in a current of air at room temperature from domestic fan. It is advisable to allow drying to proceed for at least 1 l (and it is thus convenient to have means by which the operator ca leave the dark-room).
18. The dry slides are then left for several days while the tissue 'exposes' th sensitive emulsion. The slides should be in a convenient holder (a smal slide box) contained in a light-proof box in which there is a desiccant such as calcium chloride or silica gel. The whole is best kept at about 5° C.

Exposures will vary with the situation, but when autographs were prepare of the skin of C 57 black mice with the hair follicles in the growth phase (anagen 5), exposure for 3 days gave a light dusting of silver grains over the follicles. Such preparations are suitable where grain-counting is to be carrie out. Exposures up to 21 days can be given where heavy autographs are re- quired for subjective assessment. If there is uncertainty as to the correc exposure, it is convenient to prepare three slides, exposing one for 3 days, one for 7 days, and the third for 14 days.

19. The slides are taken out of the container in the dark-room. Development is carried out for 5 min at 20° C in an amidol solution freshly made up Prepare a stable stock solution as follows:

Sodium sulphite (anhydrous)	25 g
potassium bromide	1 g
water	200 ml

Prepare developer before use from:

stock solution	10 ml
water	40 ml
amidol	0·25 g

20. The slides are rinsed with water, or taken through a photographic stop bath, and then fixed in an acid fixing bath. After this they may be brought into the light.
21. They are washed, and dried in air (compare fig. 1, B).

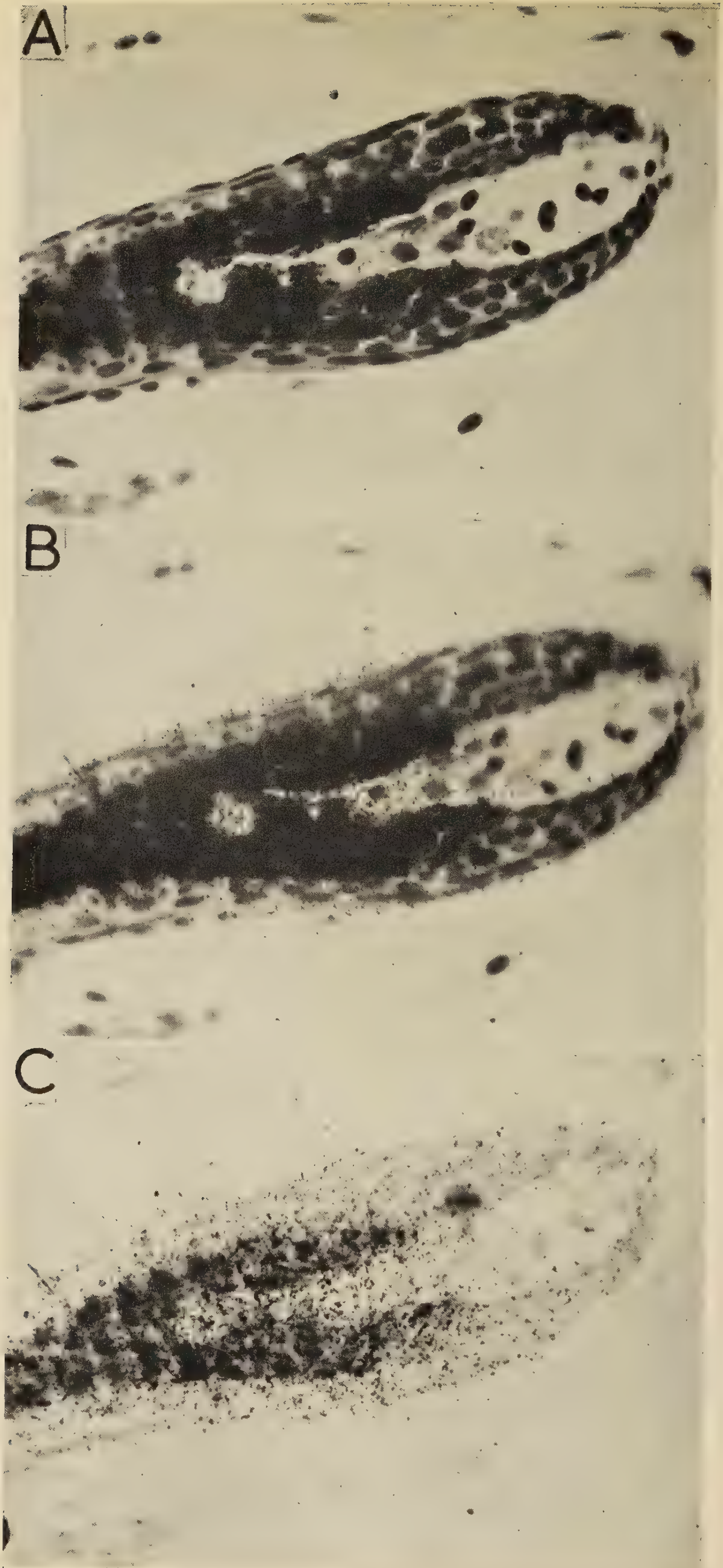

Fig. 1

The instruction leaflet supplied with Kodak AR 10 plates contains helpful nformation regarding stages 8 to 21, but there are one or two additional points vorth mentioning. With regard to cutting the film, it is most convenient to ave a 'jig' for this. Four strips of perspex can be screwed to a board, leaving oom inside them for the plate to be laid flat. If the perspex is thicker than the late, a steel rule can be laid across the plate, resting on the perspex walls, so s not to touch the surface of the plate. By using the rule as a guide, straight uts can be made with a razor blade. If pairs of screws are driven into the ase at regular intervals, the rule may be held against these, enabling a regular attern of cuts to be made. The use of scissors to cut the film, as indicated in Autoradiography' (Kodak data sheet SC-10) is not advisable.

Experience has shown that stripping takes place most easily if the plates re kept dry. Before using a box the seal of which has been broken it is advisble to have stored it in a desiccator for the previous 24 h; otherwise stripped ilm may curl badly and it cannot then be laid flat on the water surface.

Trouble has been experienced with a few plates: the film tore badly during tripping. The occurrence of a few such rogue plates seems inevitable. Tearing ccurs mostly at the periphery of the plates, and the makers' instructions to eject the outermost 1 cm of each edge of a plate should be adhered to.

The slides now bear the histological section, and the exposed and developed utoradiograph. Next the melanin is bleached, and in the process the silver rains of the radiograph are converted to salts of silver, so that, after bleaching as taken place, the silver salts must be reconverted to metallic silver by hotographic development in bright light. The tissue is then coloured with a uitable colorant. The following procedure is suggested:

2. Soak the slide in water.
3. Transfer to 4% formaldehyde and leave for 5 min.
4. Wash four times for about 1 min in tap-water; then rinse with distilled water.
5. Transfer to the bleaching solution and leave for 45 min. The solution is prepared as follows: In a suitable slide jar pour 0·2 ml concentrated hydrocholoric acid on to 0·25 g potassium chlorate. After about 30 sec add 1 ml distilled water. Loosely cover vessel to retain the gases that are

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FIG. 1 (plate). Three photomicrographs of the same hair follicle from an intense black BB, CC) mouse. The follicle was in a growth phase (Anagen 6), during which pigment is ynthesized. The tissue from which this follicle was taken had been incubated in tyrosine-2-^{14}C.

A, before radioautography. Stages 1–12 of the method had been carried out, followed by taining in haematoxylin and eosin. Melanin, appearing as very dark granules, is present in the ft half of the follicle.

B, after radioautography. Stages 1–21 had been carried out. The fine black silver grains of he radioautograph are to be seen scattered about the follicle. It can be appreciated that it ould be quite impossible to count the silver grains where the pigment is dense.

C, after radioautography, bleaching, and redevelopment. Stages 1–31 had been carried out. he melanin (and, incidentally, the haematoxylin) has been bleached, and the silver grains f the radioautograph stand out clearly against the melanin granules, which are coloured olden yellow in the preparation.

evolved. Add 100 ml water after 2 min, while swirling to dissolve th
gases.

26. Wash briefly in 4 changes of tap-water, then twice in distilled water.
27. Develop for 5 min at 20° C in the amidol solution described in section 19. This must be done in bright daylight, or with an electric lamp close to th glass vessel.
28. Rinse in tap-water, then in distilled water.
29. Transfer to Mayer's carmalum solution (Baker, 1945) and leave for 3 min
30. Rinse off excess carmalum with distilled water. Dry in air with an electric fan.
31. Transfer to 70% alcohol, pass through 90% and absolute alcohol, clear and mount in balsam or synthetic mountant. (The procedure in sections 30 and 31 is odd, but we recommend it. If the radioautograph is taken out of carmalum solution, washed, and transferred directly to alcohol (without air-drying), it often happens that vapour is trapped below the sensitive film. Once this has happened it is hard (sometimes impossible) to eradicate. After trying a number of ways of preventing this, we found the air-drying method best.)

This gives a section with the nuclei coloured pink, and in many instances the cytoplasm is hardly coloured. (If the tissue is unfamiliar, it might be advisable to use a more general stain such as a haematoxylin.) Melanin granules remain visible and are coloured golden yellow. The silver granules of the radiograph stand out black in strong contrast to the other objects (fig. 1, c).

REMARKS

When the need arose to overcome the difficulty of observing the silver grains of a radioautograph in regions of heavy pigmentation, several possible methods came to mind. Of the optical methods, dark-ground illumination, or oblique illumination (Baker, 1948), or top lighting might have served to illuminate the silver grains. However, as such optical equipment is not generally available in histological laboratories, the possibilities of success attending the use of these methods were not fully explored.

Detachment of the radiographic film from its position over the histological section and reattachment to a fresh microscope slide was considered. However, experience of handling the film showed that its fragility would make this method very difficult to carry out without causing extreme distortion. 'Contact printing' was tried: radiographic film was attached to a plain slide, and this was held in contact with a slide bearing the section to be radioautographed during the exposure period. Satisfactory radioautographs were obtained in this way, but the matching of points on the radiograph with their corresponding points on the section was unjustifiably tedious. Resort was then made to chemical methods, it being known that melanin is bleached by oxidizing agents. A number of these were used in the hope that one would be found that possessed suitable bleaching properties, yet, at the same time, did not terfere with the radioautograph. Preliminary chemical tests were carried it with 'dummy radioautographs': a slide bearing a section of pigmented ssue was treated in the solution undergoing trial simultaneously with a strip exposed and developed lantern plate. When promising results were obtained nuine radioautographs were substituted for the dummies. The following sults were obtained.

Mixtures containing potassium permanganate. Twenty volumes of a solution ntaining

potassium permanganate	3·0 g
sodium chloride	6·5 g
water	up to 500 ml

idified before use with one volume of glacial acetic acid, were used as a each. After removal of the brown stain with a 5% solution of potassium etabisulphite, the slides were rinsed in water, and redeveloped in amidol lution. Melanin was satisfactorily bleached in 20 min. The radioautographs, owever, were adversely affected. The film on occasions became detached, d generally the silver grains were rendered less visible (as would be expected, for the fluid is used as a negative reducer in photography). Slight riations in the composition of the mixture were tried with no greater success d the method was therefore not adopted.

Solutions of hydrogen peroxide of 20 volumes and less in strength were und successfully to bleach melanin, but they damaged the autoradiographic m, which became granular and often filled with blebs. The method was not rther tested.

Solutions of potassium dichromate with acid were made up as follows:

potassium dichromate, 5% aqueous solution	25 ml
concentrated hydrochloric acid from	0·3 ml
to	1·5 ml
water	100 ml

ixtures such as these are used for intensification of photographic negatives, d it was found that they had the effect of increasing slightly the size of the lver grains; however, treating the slides for periods of up to 48 h led to sufficient bleaching of the melanin (to a light brown colour).

Potassium chlorate with hydrochloric acid. Such a reagent had previously een used histologically as a bleach for melanin (Krause, 1927). Prepared as section 25 it proved to be the most satisfactory bleaching agent in the series tests. If the addition of water is omitted, bleaching will also take place when oist slides are left in the gases (chlorine and oxides of chlorine) for 15 min. he radioautographic film is not damaged by these treatments, but the silver ains are altered and redevelopment is necessary to convert them back to etallic silver.

It might be thought that so violent a reagent would attack the tissue, but e fixed tissue survives the treatment and histological details are remarkably unaltered. Even if some changes were to occur at a cytological level, this woul be of no great importance since radioautographs produced by radiation fro isotopic carbon are of low resolution: the wide angle of scatter and th marked ability of β-particles to penetrate result in the exposure of the radio graphic film at distances from the radioactive source greater than the dimen sions of most cells.

This method also has the effect of improving the radioautographs supposedl because the bleach converts the silver image to silver chloride, which o redevelopment gives better-defined silver granules than those developed i the first place from the bromide emulsion of the autoradiograph.

We wish to add this paper to the many that have been dedicated to D Gottfried Christian Hirsch in honour of his 70th birthday.

We would like to thank Professor T. B. Fitzpatrick, in whose laboratorie at the University of Oregon Medical School part of this work was carried out for the hospitality of his department and for his stimulating advice. We ar indebted to Dr. Kukita, of the University of Tokyo, in collaboration with whom the blocks were prepared that were used in developing the technique Professor Sir Alister Hardy gave encouragement and the facilities for con tinuing the work in Oxford. The generous advice of Dr. H. Greenberg o Ilford Ltd. and of Dr. H. Hirsch of Kodak Ltd. is gratefully acknowledged.

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The Mesogloea of *Pelagia noctiluca*

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With two plates (figs. 1 and 7)

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SUMMARY

The arrangement of the mesogloea fibres of *Pelagia noctiluca* is described chiefly rom material examined in the fresh state.

The role of the mesogloea in swimming is shown to be the antagonization of the ubumbrellar circumferential muscles.

The fibres of the mesogloea are shown to be collagenous by their contraction on eating, their amino-acid composition, and their appearance under the electron icroscope.

A suggestion is made to account for the characteristic arrangement of the fibres at a istance from the cellular layers.

INTRODUCTION

IN a previous paper (Chapman, 1953*a*) I attempted to show that the mesogloea, even of such transparent medusae as *Chrysaora* and *Aequorea*, contain bres and that these are of a collagen-like material. Not in all medusae are hese fibres arranged in a very striking or regular fashion but in some a pattern s clearly discernible and is related to one of the roles which the mesogloea lays in the life of the animal, namely that of antagonizing the subumbrellar uscles. A generalized diagram of this arrangement has been given elsewhere Chapman, 1958), but it was there supported by little evidence. In the present aper the structure of the mesogloea of *Pelagia noctiluca* is shown to be in ccord with the generalized diagram. The conclusion that the mesogloeal bres are of a collagen-like material has been challenged by Bouillon and andermeerssche (1957), but the results of these authors cannot be accepted their entirety, especially as collagen has been found in many invertebrates. urther experiments, supporting my previous contention and at variance with he conclusions of Bouillon and Vandermeerssche, are reported.

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The material was obtained and examined in the fresh state during a visit to Villefranche-sur-Mer.

THE DISPOSITION OF THE FIBRES

The mesogloea of *P. noctiluca* agrees with that of the hydrozoan *A. vitrina* and that of the scyphozoans *Cyanea* and *Chrysaora*, in lacking cells such as are found in *Aurelia*. It is very transparent and of firm consistency. Medusae generally do not survive long in captivity and when left to die the mesogloea is much more resistant to autolysis than the cellular layers and can easily be removed from moribund animals by rubbing off the exumbrellar ectoderm and pulling off the subumbrellar wall of the enteron with the subumbrellar muscles. In this way the mesogloeal core of the animal can be easily separated and kept for some time in sea-water. Thick hand-sections of it were cut in various planes with a razor blade and examined in sea-water, when the disposition of the fibrous elements of the mesogloea could be made out.

Near the exumbrellar surface of the mesogloea lies a plexus of very fine fibrils which run tangentially in all directions. These provide what may be called an exumbrellar 'skin' (fig. 1). These fibrils are continuous with thick fibres which run vertically between the exumbrellar and subumbrellar surfaces and which are spaced widely apart. Thus each thick vertical fibre spreads out into an increasingly finer network as it approaches the exumbrellar surface and also as it nears the subumbrella. The amount of arborization and the density of the tangential fibres is greater on the exumbrellar than on the subumbrellar side. The appearance of the fibres in a radial section of the medusa is shown in fig. 2. A section parallel with the exumbrellar plane and taken about midway between ex- and sub-umbrellar shows mainly the thick regions of the fibres with few branches (fig. 3). These can be seen to have taken a wavy or even a helical course. This suggests that in their natural state they are under some tension, which has been released when the connexions with their arborizations at the ex- and sub-umbrellar layers are severed. This, no doubt, accounts for the fact that the published figures of the mesogloea of coelenterates such as those of Tretyakoff (1937) show the mesogloeal fibres as sinuous or helical structures. In the fresh state they have only been seen to take such a course when the section has been prepared in such a way that the anchorages of the fibres with others have been broken. This has, of course, occurred in the specimen from which fig. 3 was drawn.

The branching of the vertical fibres as they approach the surfaces of the bell is very complex, and the end branches are very fine and make up the network which forms the outside 'skin' of the mesogloea. Figs. 4 and 5 are drawings of the exumbrellar and subumbrellar plexuses, from which it can be seen that so far as the tangentially running ends of the fibres are concerned there is no predominating radial or circumferential direction.

FIG. 1 (plate). Photomicrograph to show the exumbrellar plexus of fine fibres in the mesogloea of *P. noctiluca* (phase contrast).

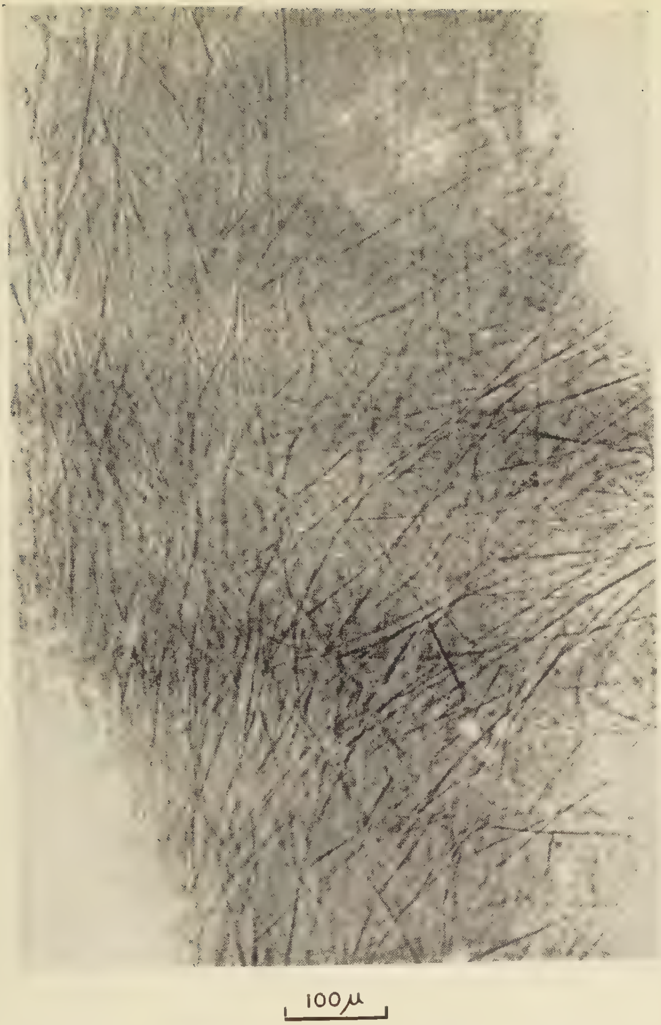

Fig. 1

G. CHAPMAN

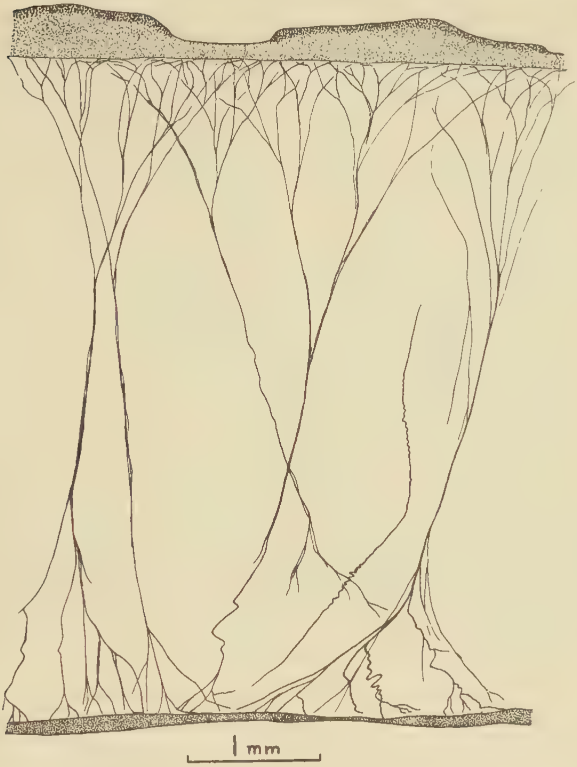

FIG. 2. Drawing of a radial hand-section of fresh *P. noctiluca* to show the thick vertical fibres and their branches. The exumbrellar surface is seen obliquely in surface view above the mesogloea and is stippled.

THE ROLE OF THE MESOGLOEA IN SWIMMING

The arrangement of the fibres of the mesogloea is consistent with their role of providing a fairly firm body to which the muscles can be attached and which will allow them to contract but will also restore them to their resting length. The musculature is almost wholly subumbrellar and is predominantly circumferential, the main coronal muscle being a band of fibres arranged very regularly side by side and covering about a third of the radius of the animal.

When swimming actively the approximate shape of the animal at its maximum contraction is shown in fig. 6, A. By comparison of this shape with that of a body of mesogloea isolated from a moribund specimen (fig. 6, B), it looks as if the mesogloea is normally somewhat distorted in the living state and at the end of a swimming contraction is maximally distorted. The truth of this obvious suggestion was tested in four ways. (1) Eight vertical, radial cuts were made in the centre of the exumbrella of an actively swimming animal. These cuts gaped. Swimming stopped for a short time but was soon resumed, although less vigorously than before. The cuts were extended to near the edge of the bell and again, after an interruption, swimming was resumed but without

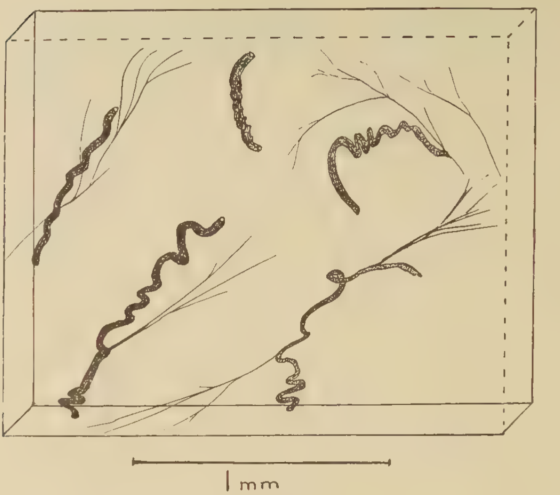

FIG. 3. Drawing of a horizontal hand-section of mesogloea of fresh *P. noctiluca* about midway between exumbrellar and subumbrellar surfaces. Five thick vertical fibres are seen somewhat obliquely. Released from their attachment to the exumbrellar and subumbrellar plexuses, they have become twisted.

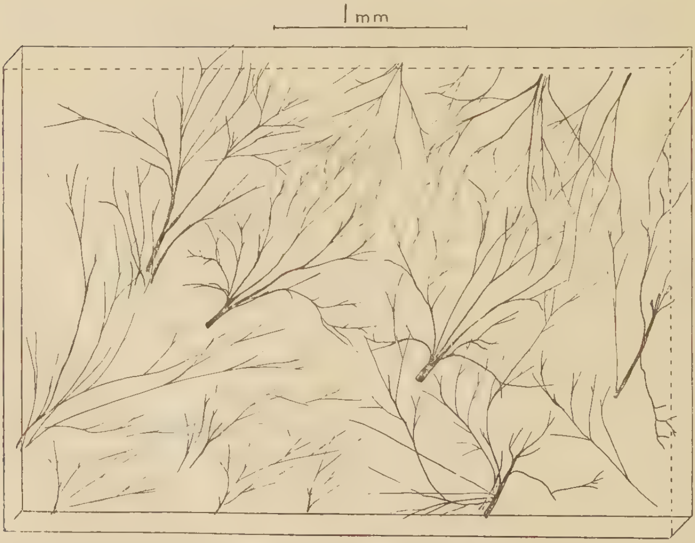

FIG. 4. Drawing of a horizontal hand-section of the mesogloea of fresh *P. noctiluca*, showing the thick vertical fibres branching to form the exumbrellar plexus. The specimen is viewed from the subumbrellar side of the thick section.

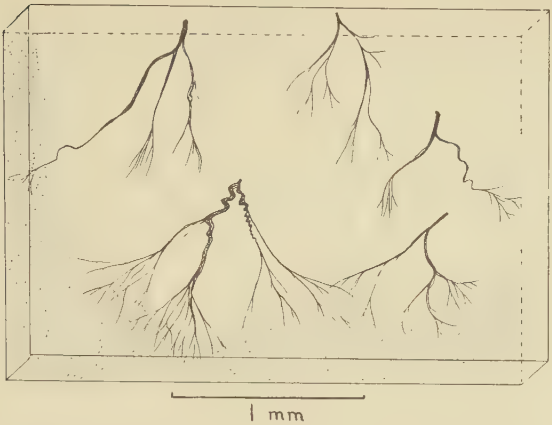

FIG. 5. Drawing of a horizontal hand-section of the mesogloea of fresh *P. noctiluca*, showing the thick vertical fibres branching to form the subumbrellar plexus. The specimen is viewed from the exumbrellar side of the thick section and traces of the endoderm, seen through the mesogloea, are indicated by stippling.

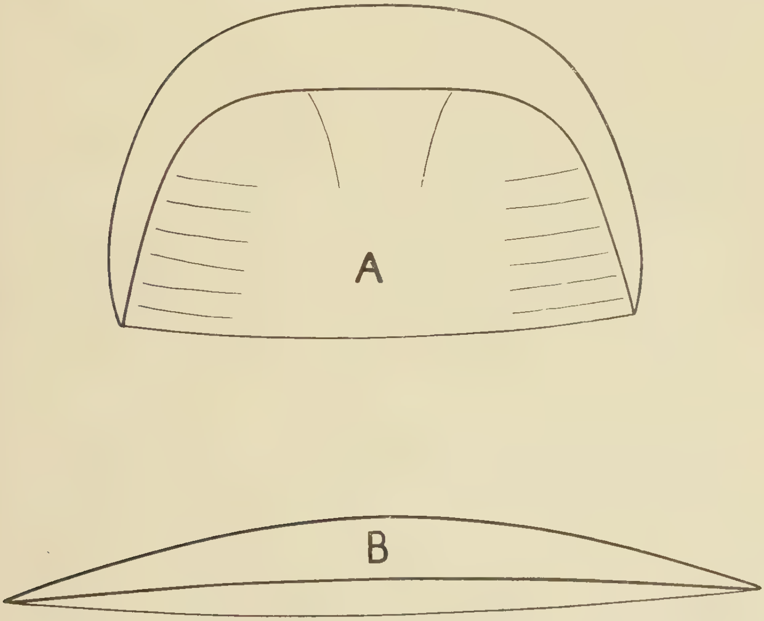

FIG. 6. Diagrams to show the relaxed and contracted shapes of the bell of *P. noctiluca*. A, fully contracted. B, fully relaxed.

the animal being able to open fully between muscular contractions. (2) A vigorously swimming medusa was placed in a dish of sea-water on the radiato and left to warm slowly. Before the temperature had quite reached 30° C muscular contractions stopped and the animal took on a flattened shape as seen in fig. 6, B. At temperatures below 30° C contraction of the mesogloeal substance does not occur (see p. 605), so there is little doubt that the change in shape was due to the relaxation of the subumbrellar muscle only. (3) The oral arms of an active medusa were removed and swimming was soon resumed with unimpaired vigour. The effect of the subumbrellar muscles was then partly destroyed by making 8 radial cuts through them. The form taken up by the animal was much flatter than that of an intact animal. Co-ordinated swimming movements were never resumed although unco-ordinated contractions of the sections of the muscle were made even 24 h after the experiment was begun (4) Finally, a medusa was anaesthetized in a mixture of equal volumes of sea-water and $7\frac{1}{2}\%$ $MgCl_2.6H_2O$ solution in water. After 2 h it was relaxed and flattened, but still responded to touch. After 14 h its condition was unchanged except that it no longer responded to stimulation.

These four experiments and observations of the shape and consistency of the isolated mesogloea leave no doubt that its 'natural' shape is close to that of a plano-convex lens and that it is distorted by the contraction of the subumbrellar muscle. This distortion stretches the exumbrella and probably also the vertical fibres, and it is the tensions which are generated in the whole mesogloea that restore the shape of the bell for the next swimming contraction.

THE NATURE OF THE FIBRES

I presented evidence previously (1953*a*, *b*) which seems to support the view that the fibres of various coelenterates are of a collagen-like material Evidence for this view has been accumulating since Astbury's (1940) announcement that the X-ray diffraction pattern of material obtained from a jellyfish resembled that of vertebrate collagen. In his survey of the occurrence of collagen and chitin, Rudall (1955) expresses no doubt that collagen is found as the chief structural protein in coelenterates. This has been denied by Bouillon and Vandermeerssche (1957) as a result of work which they did on *Pelagia*, *Aurelia*, and *Limnocnida*. They regard the mesogloeal fibres as being composed of elastin. Unfortunately there is no simple test for the collagens but they are characterized by several physical and chemical features which when taken together are not found in other proteins. Although elastin is now regarded as a member of the collagen group as opposed to the keratin–myosin–epidermin–fibrin group and shares certain properties with collagens (and particularly with collagen that has undergone thermal contraction), there are properties in addition to tinctorial behaviour in which collagen and elastin are different. The main ones of these are listed below.

Collagen	*Elastin*
Usually banded at 640-Å intervals when seen in electron micrographs	Not banded
Contracts on heating	Does not contract
X-ray diffraction pattern characteristic	X-ray diffraction pattern 'amorphous'
High content of hydroxyproline	Low content of hydroxyproline
Not stained by orcein	Stained by orcein
Stained pink by Weigert's connective tissue stain	Stained blue-black by Weigert's stain

Even if the distinction between collagen and elastin does not seem nowadays to be quite so clear as it was thought to be, the differences tabulated above are sufficient to make the distinction reasonably sharp. It will also be recalled that, so far, Bouillon and Vandermeerssche appear to be alone in claiming to have identified elastin in an invertebrate, almost all the references in the literature being to the elastin of mammals.

A fibrous protein is generally accepted as a collagen if it shows several of the characters tabulated above and especially if its hydroxyproline content is high. It is true that there is considerable variation in this value among invertebrate collagens (Watson, 1958) and that it is, in general, lower than in vertebrate material. Nevertheless, it has not yet been shown to fall as low in a recognizable invertebrate collagen as it does in mammalian elastin.

Bouillon and Vandermeerssche (1957) claim that the mesogloeal fibres are unaltered by dilute acetic acid, that they undergo no structural modification when boiled, and that they are partially digested by trypsin. They state that electron micrographs show that the fibres are uniform like those of mammalian elastin and not banded like those of collagen. Finally, they claim that the X-ray diffraction pattern resembles that of 'amorphous' material like elastin and not the characteristic pattern of collagen. The difficulties of preparation of a substantial amount of orientated fibres from the mesogloea of medusae for X-ray examination suggests, perhaps, that not too much reliance can be placed on this test in practice, particularly if it shows a negative result.

Some of the experiments have been repeated on *Pelagia* only, with results which do not accord with theirs but which instead serve to confirm the contention that the mesogloea is of a collagenous nature.

Changes on heating

There is no doubt that the mesogloea of *Pelagia* undergoes marked changes of shape and size when it is heated in distilled water and in sea-water. An isolated mesogloea was cut in half and one half was heated slowly in sea-water from room temperature (about 20° C) to 60° C, at which it was kept for 5 min. The whole experiment occupied about 20 min. No contraction took place below 30° C. It was difficult to be certain whether or not contraction began below 40° C, but there is no doubt that between 40° and 50° C it took place and was rapid as the temperature approached 60° C. Both halves of the mesogloea were preserved in dilute formalin. A photograph of them is given in fig. 7, A. The experiment was repeated several times with the same results.

The experiment was repeated on a specimen of *Pelagia* which had been preserved for several days in dilute formalin. No change of size took place when the specimen was heated in sea-water until the temperature had risen to between 80° and 85° C. On boiling for 3 min a considerable contraction took place, the amount of the contraction being a change of diameter from 6 cm to 1 cm. The consistency of the material became more gelatinous and less firm. Microscopic examination of the contracted mesogloea showed that the vertical arborescent fibres were much shorter, but they were still distinguishable and had not become disorganized or converted into gelatin.

Bouillon and Vandermeerssche do not state whether the material which they used for tests of thermal contractility was fresh or preserved, but the remark that it was *collected* at Villefranche-sur-Mer suggests that their experiments were made on preserved material. If that is indeed so, it was thought that it might account for the lack of visible structural modification which they report. Therefore a further experiment was made on material which had been kept in 5% formalin for 7 months. However, this test yielded the same result as that obtained with material preserved for only a few days. No marked contraction began below about 80° C, but at this temperature contraction was rapid and the diameter of a half medusa was reduced from about 6 cm to 3 cm in 5 min. Subsequent boiling caused a further contraction and the diameter became about 2·5 cm. A photograph of the two halves is given in fig. 7, A. It is very difficult to understand how Bouillon and Vandermeerssche came to the conclusion that no change in the mesogloea occurred on boiling.

If the change which took place on heating the mesogloea were simply the loss of the ground substance between the fibres, one would expect the material to change in consistency but not in size, unless the fibres are normally held in a very much stretched state by the presence of the matrix. If the fibres were stretched in this way they should become sinuous on the loss of the matrix, as has already been noted to occur sometimes in fixed material. But the fibres of the heated material do not become sinuous; so it must be concluded that it is they that have shortened, as do the mesogloeal fibres of *Calliactis* (Chapman, 1953*a*).

Effect of dilute acetic acid

The Belgian authors state that the mesogloea of *Pelagia* and other medusae is not altered by immersion for 48 h in 1% acetic acid. My experiments on

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FIG. 7 (plate). A, photograph of untreated halves and boiled halves of *P. noctiluca*. *a*, *a*, experiment performed on isolated fresh mesogloea, which was afterwards preserved in 5% formalin. *b*, *b*, experiment performed on material preserved in 5% formalin for 7 months. A whole animal without the oral arms was used.

B, electron micrograph of fibres of the mesogloea of *P. noctiluca*. A fragment of the tissue from the centre of the bell was homogenized and stained with phosphotungstic acid. (Electron micrograph by Dr. Jean Hanson.)

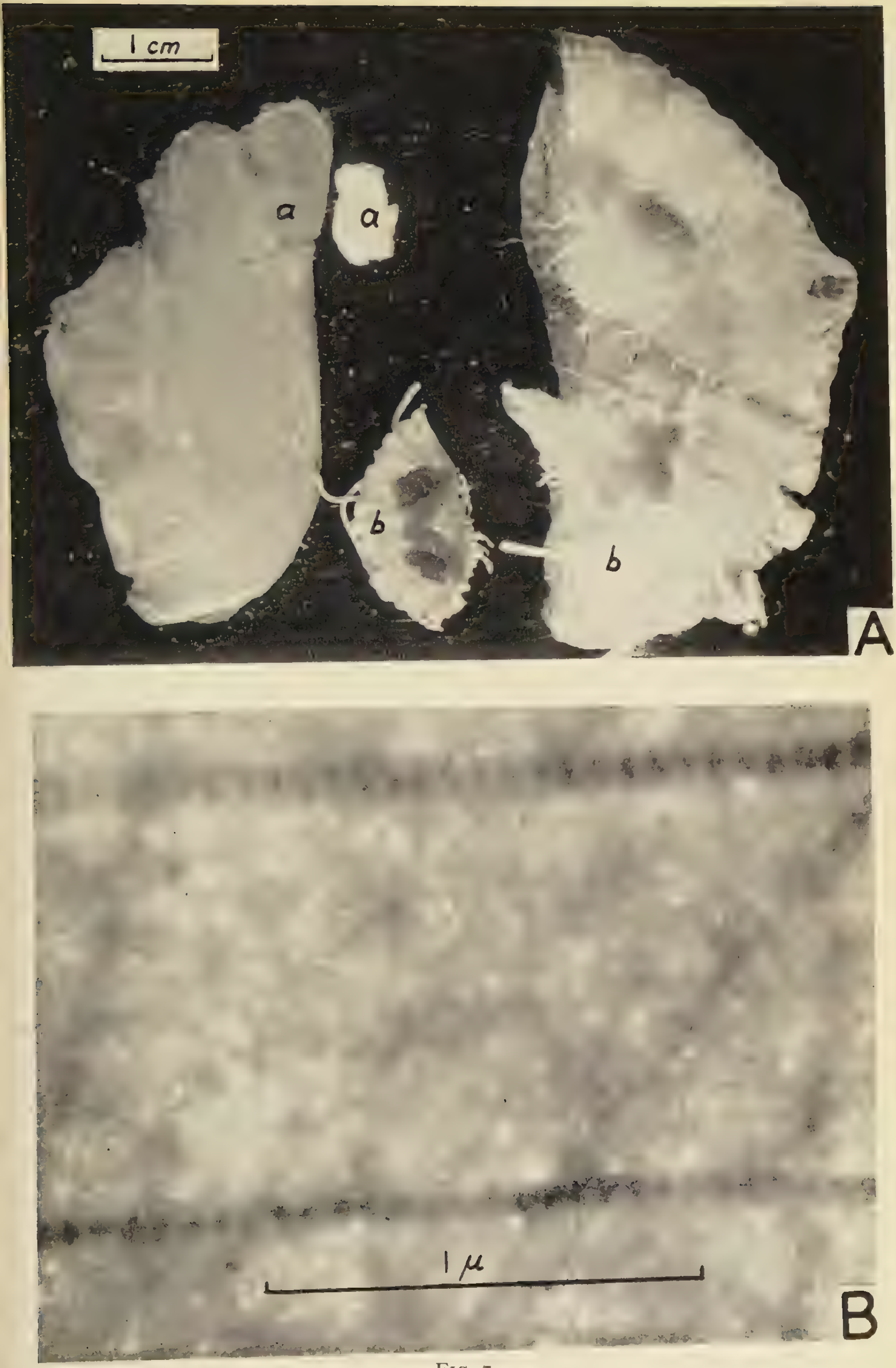

FIG. 7
G. CHAPMAN

fresh, isolated mesogloea are not in accord with this. Some pieces of isolated mesogloea were placed in distilled water and some in 1% acetic acid. After 48 h the consistency of the specimens kept in water differed from that of the acid-treated ones. The specimens in distilled water retained their original firmness while those in acid were more gelatinous. No clear difference in the appearance of the fibres could, however, be seen under the microscope between the treated and untreated specimens.

After 96 h the difference in consistency between the treated and untreated specimens was more marked, that in the distilled water being little changed from its original state, while that in the acetic acid was soft and gelatinous but possessed sufficient coherence to return to its original length when stretched with needles to twice its length. The control specimen broke when stretched with needles, as does fresh mesogloea.

Electron microscopic examination

The distance between the well-known striations of the collagen fibre varies according to the source of the material and may lie between 600 and 700 Å, but is generally about 640 Å. Some collagens, such as those of earthworm cuticles, are not striated. The distance of 640 Å would be represented by 0·5 mm in the photograph published by Bouillon and Vandermeerssche. The definition of this picture and the insufficiency of the magnification at which it was taken may well throw doubt on their contention that the photograph is a clear demonstration of the unstriated nature of the fibres of the mesogloea of *Pelagia*.

A fragment of the mesogloea of *Pelagia* preserved in formalin was treated in a homogenizer for 1 min, stained with phosphotungstic acid, and examined at a magnification of 20,000 in the electron microscope. There is no doubt that the fibres are banded, and measurements made on photographs of the specimens indicate that the periodicity is about 660 Å, which lies within the limits of the periodicity of invertebrate collagens. An electron micrograph of the mesogloeal fibres of *Pelagia* is shown in fig. 7, B.

Amino-acid composition

For a protein to be characterized as collagen it should possess not only thermal contractility and banded structure of the right periodicity, but also a high hydroxyproline content.

Pieces of *Pelagia* mesogloea were cut from the centre of the bell and carefully examined to ensure that they were free of cells and composed of mesogloea only. They were preserved in 70% alcohol, in which they were kept for 8 weeks. They were dehydrated in absolute alcohol (3 changes), washed with ether, and allowed to dry in the air and finally at 105° C overnight. The total nitrogen was estimated by a micro-Kjeldahl method and amounted to approximately 10·09% of the dry residue. This is lower than is normally found in collagen, but it may well be accounted for by the lack of washing in water to remove inorganic salts. Hydroxyproline was determined by the method of

Neuman and Logan (1950) and was found to be 3·6% of the dry residue, which is a much lower percentage than is found in vertebrate collagen or in earthworm cuticle and lower than that found by Gross, Sokal, and Rougvie (1956) in *Spongia graminea*, but not a great deal lower than the 4·41% found in the ejected filaments of *Holothuria forskali* by Watson (1958).

I was unable to stain the mesogloea of fibres of formalin-fixed *Pelagia*, a blue-black colour in Weigert's connective tissue stain, nor did they stain with orcein. Elastin fibres in sections of rabbit aorta which were treated alongside the sections of *Pelagia* stained readily.

DISCUSSION

There is now considerable evidence that collagen is found in animals belonging to most invertebrate phyla (Rudall, 1955), but there is equally no doubt that it varies considerably in its chemical composition (Watson, 1958), so that a range of structural proteins can be called by the name, some being banded and others being plain, when seen in electron micrographs. Their identification still depends on the collection of several items of evidence, although there is also a tendency (Melnick, 1958) to regard any structural protein as collagen if it contains hydroxyproline. The evidence from X-ray diffraction was the first to be used to identify collagen in the Coelenterata (Astbury and Bell, 1939; Astbury, 1940; Champetier and Fauré-Fremiet, 1942; Marks, Bear, and Blake, 1949); but obtaining a negative result, as Bouillon and Vandermeerssche appear to have done, cannot really be regarded as proof that no repeated structure exists and that the material is therefore amorphous and therefore not collagen. They give no details of how they prepared their material for X-ray analysis and it is, perhaps, not surprising, unless they took great pains to obtain a specimen with parallel fibres, that they did not succeed in revealing the presence of collagen by this method.

Bouillon and Vandermeerssche mention briefly the role of the mesogloea in the swimming movements of medusae and state that the bell regains its original form after muscular contraction owing to the elasticity of the mesogloea, but they conclude that the elasticity is due to the physical properties of elastin, since collagen, they say, is inextensible. The mesogloea is, however, a complex structure of fibres and matrix and it may well be that the role of the fibres is to transmit the tensions set up by the subumbrellar muscles so that the matrix as a whole is distorted. It is not merely that tension is set up which stretches the fibres.

Bouillon and Vandermeerssche are agreed that the fibres of the mesogloea are not dependent for their origin on the presence of cells in the mesogloea, and they look to the region of mesogloea at the base of the endoderm especially as the site of formation where, they point out, it is relatively strongly stained by the Hotchkiss–MacManus method for polysaccharides. It is indeed likely that the fibres originate in this region and, as is now well known, mucopolysaccharides are almost always associated with collagen. Gross (1956) has own how various conditions, including the presence of mucopolysaccha-des, can bring about the precipitation of the collagen from solution. There eems good reason to suppose that the endodermal and ectodermal cells both ecrete the precursors of mesogloea substances at their base and that the fibres hich arise from the material are orientated according to the stresses to which ey are subjected during their growth. Although there is at present no vidence in support of it, it is tempting to suggest that the formation and rowth of the mesogloea fibres takes place in something of the following anner.

The finest fibrils would be formed near to the cells of ectoderm and endo-erm and from material secreted by them; they would have a random orienta-ion. In the youngest animals, where ectoderm and endoderm are very close ogether, some fibres will doubtless stretch from one cellular layer to the other. s growth takes place and mucopolysaccharides are secreted, and as the bulk f the animal increases, the cellular layers are moved apart. Those fibres hich chanced to run from the subumbrella to the exumbrella will come to e on the shortest path joining their points of attachment to the surface and hey will tend to pull out other fibrils attached to them from over a wider area o that each fibril will be attached to the base of the epithelium over quite a ide area by numerous very fine fibrils which run into the surface 'skin' of ibrils. It would be interesting to know if, in fact, this is the way in which the attern of mesogloea fibres is formed and, since there appear to be no pub-ished papers dealing with the subject, it is hoped that an investigation will hortly be undertaken.

I cannot speak too highly of the helpfulness of the Director and Staff of the Zoological Station at Villefranche-sur-Mer. I am indebted to the Central Research Fund of the University of London for a grant which made the visit to the Station possible. The chemical analyses of total nitrogen and of hydroxy-proline were very kindly done for me by Mr. M. R. Watson of the M.R.C. Biophysics Research Unit, King's College, London. Dr. Jean Hanson kindly took the electron micrograph of mesogloeal fibres shown in fig. 7, B.

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